

THESIS

1955
STOCKHOLM

ACTA RADIOLOGICA
SUPPLEMENTUM 125



ROENTGEN
ABSORPTION SPECTROPHOTOMETRY
IN QUANTITATIVE CYTOCHEMISTRY

BY

BO LINDSTRÖM

DANE MEDICAL LIBRARY
STANFORD UNIVERSITY
300 PASTEUR DRIVE
PALO ALTO, CALIF.

Diss. med.

Stockholm. Karolinska Institutet

12 MAJ 1955

STOCKHOLM 1955

This volume which forms Supplement 125 to Acta Radiologica
may be obtained from

ACTA RADIOLOGICA, STOCKHOLM 2, SWEDEN

or the usual agents

FROM THE INSTITUTE FOR CELL RESEARCH, MEDICAL NOBEL INSTITUTE, KAROLINSKA
INSTITUTET, STOCKHOLM, SWEDEN.

ROENTGEN
ABSORPTION SPECTROPHOTOMETRY
IN QUANTITATIVE CYTOCHEMISTRY

BY

BO LINDSTRÖM

STOCKHOLM 1955

Printed in Sweden

Tryckeri Aktiebolaget Thule

Stockholm 1955

550240

Acknowledgements

The present investigation has been carried out at the Institute for Cell Research at the Medical Nobel Institute, Karolinska Institutet, Stockholm.

To Professor Torbjörn Caspersson, head of the Institute, I wish to express my deep gratitude for his unfailing interest in this work and for his generous support throughout this investigation.

I am greatly indebted to Professor Arne Engström, head of the Institute for Physical Cell Research, for introducing me in the field of histochemical roentgen analyses, for the stimulating collaboration, for his valuable advice and for many helpful discussions during my early years at the Institute.

I am very grateful to Docent Gunnar Moberger for stimulating collaboration and valuable discussions at the biologic application of the roentgen absorption methods.

My sincere thanks go to Engineer Lars Andersson for his valuable and untiring technical assistance throughout this investigation.

I want to thank Miss Barbro Sörensson for her able assistance in preparing the specimens for the roentgen absorption analyses and for preparing the manuscript.

Engineer Lars Wegstedt has given valuable technical assistance at the construction of the high voltage supply for the roentgen spectrophotometer for which I am very grateful.

My thanks also go to the staff at the workshop at the Institute, where the roentgen tubes and the roentgen spectrophotometer were built.

This investigation has been financially supported by Statens Naturvetenskapliga Forskningsråd, the Swedish Cancer Society, Stiftelsen Therese och Johan Anderssons Minne, and Karolinska Institutet (reservationsanslaget), for which I want to express my deep gratitude.

Stockholm, April 1955

B o L i n d s t r ö m

*To
my father
and to
the memory of
my mother*

Table of contents

Introduction	9
 Part I. The Physical Basis of Roentgen Absorption Spectrophotometry in Quantitative Cytochemistry	
Chapter 1. The Production of Roentgen Rays	15
A. The Continuous Roentgen Spectrum	15
B. The Characteristic Roentgen Spectra	22
Chapter 2. The Absorption, Scattering, and Refraction of Roentgen Rays	26
A. The General Laws for Absorption	26
B. The Scattering Process	28
C. The Photoelectric Absorption Process	29
D. The Absorption Edges	31
E. Empirical Absorption Formulae and the Magnitude of the Absorption Edges	35
F. The Refraction of Roentgen Rays	40
Chapter 3. The Production of Monochromatic Roentgen Rays	43
A. Methods Based upon the Filtration of the Roentgen Beam	43
B. The Diffraction of Roentgen Rays by Crystals; Bragg's Law	45
C. Roentgen Spectrographs with Bent Crystals	47
D. The Diffraction of Roentgen Rays by Ruled Gratings	50
Chapter 4. The Measurement of the Intensity of Roentgen Rays	52
A. The Photographic Action of Roentgen Rays	52
B. Methods Based upon the Ionizing Effect of Roentgen Rays on Gases	55
C. A Method Based upon the Fluorescent Effect of Roentgen Rays	57

Part II. **Total Dry Weight Determinations of Cytologic Structures by Absorption Spectrophotometry Utilizing Soft Continuous Roentgen Radiation**

Chapter 5. Earlier Biologic Applications of Microradiography with Special Reference to Total Dry Weight Determinations ..	61
A. Microradiography with Secondary Magnification ..	61
B. Microradiography with Primary Magnification	62
C. Quantitative Microradiographic Determination of the Total Dry Weight of Small Biologic Objects	63
Chapter 6. The Theoretical Basis	67
A. Mass Absorption Coefficients of the Elements with Low Atomic Numbers and of Some Common Organic Compounds in Biologic Material	67
B. The Systematic Errors Utilizing Monochromatic Roentgen Radiation	72
C. The Systematic Errors Utilizing a Continuous Roentgen Spectrum	85
D. The Reference System and the Final Calculation of Weight per Unit Area	90
Chapter 7. The Experimental Apparatus	94
A. The Roentgen Tube	94
B. The Vacuum System and the High Voltage Supply ..	100
Chapter 8. The Experimental Procedure	103
A. Statistical Methods	103
B. The Preparation of the Specimen	104
C. The Procedure of Making the Reference System and Its Accuracy	105
D. The Photometric Procedure	107
E. The Spatial Resolving Power of the Microradiographic Technique	111
F. The Quantitative Resolving Power of the Microradiographic Technique	114

G. The Total Resolving Power and the Combined Experimental Error of the Quantitative Microradiographic Technique	118
Chapter 9. Applications	122
 Part III. Elementary Analyses of Small Biologic Objects by Quantitative Roentgen Absorption Spectrophotometry	
Chapter 10. Survey of Quantitative Elementary Analyses by Roentgen Ray Methods with Special Reference to Biologic Applications	133
A. Quantitative Roentgen Ray Fluorescence Analyses ..	133
B. Quantitative Elementary Analyses of Chemical Compounds or Mixtures by Roentgen Absorption Spectrophotometry	134
C. Elementary Analyses of Biologic Objects by Quantitative Roentgen Absorption Spectrophotometry	135
Chapter 11. The Theoretical Basis for Elementary Analyses by Roentgen Absorption Spectrophotometry	137
A. The Principle of the Method	137
B. The Accuracy and the Sensitivity of the Method ..	139
Chapter 12. The High Vacuum Roentgen Ray Spectrophotometer	143
A. The Choice of Experimental Apparatus for Elementary Analyses	143
B. The Principle of the Spectrophotometer	143
C. The Body of the Spectrophotometer	144
D. The Crystal Holder and the Crystal Table	149
E. The Crystals	150
F. The Registering System	150
G. The Vacuum System	157
H. The High Voltage Supply	159

Chapter 13. The Experimental Procedures	165
A. The Adjustment of the Spectrophotometer	165
B. The Selection of Suitable Monochromatic Radiation for Elementary Analyses	166
C. The Influence of Broadening Effects Inherent in the Bent Crystal Technique	167
D. Registering with the Geiger Counter	168
E. The Photographic-Photometric Procedure	170
F. The Error of the Photographic-Photometric Procedure	176
Chapter 14. Applications of Elementary Analyses on a Cellular Level	178
A. Determination of Calcium and Phosphorus in Osseous Tissue	178
B. Determination of Calcium and Phosphorus in the Arterial Wall in Arteriosclerosis	183
C. Determination of Calcium in a Renal Calculus	183
D. Determination of Phosphorus in Sea Urchin Eggs ..	191
E. Determination of the Amount of Sulphur in Rat Epidermis	191
General Summary	193
References	197

Introduction

In histo- and cytochemistry physical procedures have become of increasing importance among the analytic methods, especially among those with a quantitative aim. In these branches of biologic and medical research the range of dimensions of the structures to be investigated is the critical factor. The upper limit of the dimensions of the structures to be analysed is generally between 10 and 20 μ . Owing to the inhomogeneity and complexity of the cytologic material, however, a linear resolution of at least 1 to 2 μ is desirable, when a *direct* analytic procedure is applied, i.e. when the analyses of cytologic structures are performed *in situ*. With such a resolution the volumes of tissue analysed are of the order of magnitude of 5 to 100 μ^3 , corresponding to a weight of between 5 and 100 $\mu\mu\text{g}$ ($1 \mu\mu\text{g} = 10^{-12} \text{g}$) for a tissue with a density of about $1 \text{g}\cdot\text{cm}^{-3}$. As the average content of water in tissues is between 70 and 80 per cent, the lower limit of the amount of dry material analysed is between 1 and 30 $\mu\mu\text{g}$. For the quantitative application of direct analytic procedures the localization and identification of the structures present no great difficulties, but as only extremely small amounts of material are available for analyses, the same relative accuracy as for microchemical methods in general cannot be expected.

The other possibility of performing cytochemical analyses is to combine isolating procedures, such as micro-manipulation and differential centrifugation, or extraction procedures, with chemical methods in the ultra micro-scale. Great difficulties are often involved in the localization and exact definition of the biologic material analysed. As these *indirect* analytic procedures can only rarely be performed on a single cell or a small number of identical cells, the analyses are generally obtained as average values for a certain amount of biologic material, and the results can rarely be related to the metabolic unit of the organism, the individual cell. Furthermore, tissues are composed of cells from various origins and with different qualities. Therefore, individual characteristics of a small group of cells can be concealed, even if the indirect cytochemical procedures are applied to extremely small volumes of tissue.

In cytochemical investigations several mutually independent, direct and indirect methods ought to be utilized to elucidate the different problems. The

results must then be compared and evaluated with respect to the experimental errors and the inherent uncertainty of the different methods.

Among the direct analytic cytochemical methods *quantitative absorption spectrophotometry* in the ultra micro-scale, utilizing different regions in the electromagnetic spectrum, plays an important rôle. The pure rotational absorption spectra of molecules in the *far infrared* region at wavelengths longer than about $100\ \mu$ cannot be applied as a direct cytochemical method because of insufficient spatial resolution. For the same reason the vibration — rotation absorption spectra of molecules in the *near infrared* region at wavelengths from about $100\ \mu$ down to $2\ \mu$ has a restricted applicability as a direct method in cytochemistry.

In the *visible* and *near ultraviolet* regions, i.e. at wavelengths from about $8000\ \text{\AA.U.}$ to $2000\ \text{\AA.U.}$ ($1\ \text{\AA.U.} = 10^{-4}\ \mu$), the molecular absorption is caused by transitions of outer electrons within the molecules with superimposed molecular vibrations and rotations of the molecules. Sometimes the ultraviolet radiation quanta have enough energy to eject an outer electron from a molecule, i.e. to ionize the molecule. In these wavelength regions the attainable resolution and the transmission conditions, i.e. the magnitude of the extinction coefficients and the range of concentrations of different organic compounds, are favourable for direct cytochemical analyses on thin sections or smear preparations of the biologic material. Extensive cytochemical investigations have been performed on different organic compounds with characteristic absorptions between 8000 and $2000\ \text{\AA.U.}$, i.a. on the cyclic amino acids, the purines, and the pyrimidines in the ultraviolet region [11, 12]. Staining reactions, enzymatic digestion methods, and extraction procedures have also been combined with direct absorption spectrophotometry in the ultra micro-scale, utilizing these wavelength regions. When these additional procedures are applied on biologic material *in situ*, however, their specificity must be carefully analysed.

In the *vacuum ultraviolet* spectral region from $2000\ \text{\AA.U.}$ to about $100\ \text{\AA.U.}$ the transmission of radiation is very small even in extremely thin sections of a biologic specimen, which limits the possibilities of performing accurate absorption spectrophotometry on biologic material *in situ*.

The *soft roentgen* region overlaps with the short wavelength part of the vacuum ultraviolet region, and extends from about 150 — $100\ \text{\AA.U.}$ down to about 2 or $1\ \text{\AA.U.}$ In this part of the electromagnetic spectrum, as compared with the vacuum ultraviolet region, the absorption of radiation in thin biologic

objects has decreased to such an order of magnitude that absorption spectrophotometry, utilizing soft roentgen radiation, can be applied *in situ* in cytochemical investigations. Utilizing the absorption of monochromatic roentgen radiation, quantitative elementary analyses have been performed on a histochemical scale [29], and a method to determine the total dry weight of small biologic objects, based upon the absorption of soft polychromatic roentgen rays, has been presented [30].

In the *ordinary roentgen* region, i.e. roentgen radiation with wavelengths shorter than about 1 or 2 Å.U., the progressively smaller absorption of roentgen radiation at decreasing wavelengths precludes quantitative absorption spectrophotometry on a cytochemical scale. Bone and other calcified tissues are exceptions, as an adequate absorption is obtained also in thin layers of these specimens, using ordinary roentgen radiation, which is absorbed to a very small extent in thin layers of soft biologic material.

The aim of the present work has been to increase the resolving power and the accuracy of the two cytochemical methods, based upon quantitative roentgen absorption spectrophotometry, *to make possible elementary analyses on a cellular level, expressed as percentage of weight*, independent of the thickness of the specimen and of shrinkage effects. The theoretical backgrounds of the two methods have first been revised, the different sources of errors have been investigated, and the possibilities to increase the resolving power have been considered. New experimental equipment has been constructed for total dry weight determinations and elementary analyses on a cytochemical scale. The experimental procedures have then been developed and tested, and certain applications of the two methods have been made.

The presentation of this work is divided into three parts. The first part comprises the physical background of roentgen absorption spectrophotometry in quantitative cytochemistry. The cytochemical applications of quantitative roentgen absorption spectrophotometry, i.e. the methods of total dry weight determinations and elementary analyses on a cellular level, are presented in the second and third parts.

PART I

The Physical Basis of Roentgen Absorption Spectrophotometry in Quantitative Cytochemistry

CHAPTER I

The Production of Roentgen Rays

In Chapters 1—4 a short review will be given of those parts of the physics of the roentgen rays which are of importance for roentgen absorption spectrophotometry applied in quantitative cytochemistry. For a detailed discussion of the physics of roentgen rays and especially for the derivations of the basic formulae reference should be made to the textbooks of roentgen physics, e.g. [34, 53, 73, 88, 16, 91, 37, 15].

Electrons are emitted from the hot filament cathode in the roentgen tube and are accelerated in the electric field between the anode and the cathode. When the electrons (cathode rays) hit the anode a very small fraction of their total kinetic energy is converted into roentgen radiation, whereas the greatest part of the energy is dissipated in the form of heat. The *efficiency* η of a roentgen tube, i.e. the fraction of the energy of the cathode rays converted into roentgen energy, is approximately given by

$$\eta \approx 1.4 \cdot 10^{-9} ZV \dots\dots\dots (1),$$

where Z is the atomic number of the element constituting the anode, and V the potential difference in volts between the anode and the cathode [91, p. 14]. In the roentgen tube used for total dry weight determinations, to be described later, platinum ($Z=78$) has generally been used as target material with a voltage across the tube of between 1000 and 1500 volts during most of the experimental investigations. According to equation (1) the efficiency is then only between 0.011 and 0.016 per cent, but at these low voltages the validity of the expression has been questioned.

A. The Continuous Roentgen Spectrum

Polychromatic roentgen rays, constituting a *continuous roentgen spectrum*, are always generated by the incident cathode rays when these are retarded at the atoms of the anode. The distribution of the intensity in this continuous spectrum and the wavelength range are independent of the target material.

Some of the physical qualities of roentgen radiation cannot be explained

unless a beam of roentgen rays is composed of discrete packets of energy, called roentgen quanta or photons, which is postulated in the quantum theory. Each roentgen quantum is generated in one elementary process, i.e. the energy of a roentgen quantum corresponds to the whole of or a fraction of the kinetic energy of *one* incident electron. If an electron loses its total kinetic energy in one process of retardation at the anode, and if this energy is quantitatively converted to roentgen radiation, *the maximum energy of a generated roentgen quantum* is obtained. For a potential difference V_{esu} between the anode and the cathode, this maximum energy is given by

$$V_{\text{esu}}e = h\nu_0 \quad \dots\dots\dots (2).$$

V_{esu} = the voltage applied to the roentgen tube (electrostatic units). 1 esu for potential difference = 299.796 volts (absolute) [46].

e = the charge of an electron = $4.8029 \cdot 10^{-10}$ esu [25].

h = Planck's constant = $6.6252 \cdot 10^{-27}$ erg · sec [25].

ν_0 = the frequency (sec^{-1}) of the roentgen quantum with maximum energy.

As roentgen radiation is an electromagnetic wave motion the following relation holds between the frequency, the wavelength, and the velocity of a roentgen quantum:

$$\nu\lambda = 10^8 c \quad \dots\dots\dots (3).$$

ν = the frequency of the roentgen quantum (sec^{-1}).

λ = the corresponding wavelength of the roentgen quantum (Ångström units).

1 Å.U. = 10^{-8} cm.

c = the velocity of light in vacuum = $2.99793 \cdot 10^{10}$ cm · sec^{-1} [25].

From equations (2) and (3) the minimum wavelength λ_0 of the generated continuous roentgen spectrum, corresponding to the maximum frequency ν_0 , can be calculated.

$$\lambda_0 = \frac{12.398}{V_a} \quad \dots\dots\dots (4),$$

where λ_0 is expressed in Å.U., and V_a is the voltage across the tube in absolute kilovolts.

From a practical point of view the units in expression (4) ought to be converted in two respects. Based on the International ohm and ampere 1 International volt corresponds to 1.00045 absolute volts [46]. The wavelength scale in the roentgen region is based upon the effective calcite grating space

at 18° C at diffraction in the first order, which is defined as 3029.04 X.U. [88, pp. 43—44]. From absolute determinations of wavelengths of roentgen emission lines with ruled gratings (see Chapter 3:D) a systematic error of the calcite grating space has been found. Therefore 1000 X.U. = 1 kX.U. is not exactly 1 Å.U. but corresponds to 1.00206 Å.U. [25], which is expressed in the following ratio between the two scales.

$$\frac{\lambda_{\text{grating (X.U.)}}}{\lambda_{\text{crystal (X.U.)}}} = 1.00206 \cdot 10^{-3} \quad \dots\dots\dots (5).$$

As far as possible the wavelength unit based on the crystal scale, i.e. X.U. or kX.U., will be used in the following presentation, as the wavelengths of roentgen emission lines are generally expressed in this unit in the physical literature. If λ_0 is expressed in kX.U. and V is the voltage across the tube in International kilovolts (kV), expression (4) takes the following form:

$$\lambda_0 = \frac{12.367}{V} \quad \dots\dots\dots (4a).$$

If the wavelength is expressed in kX.U., equation (3) is changed to

$$\nu\lambda = \frac{10^8 c}{1.00206} \quad \dots\dots\dots (3a).$$

The short wavelength limit of the continuous roentgen spectrum, called the “Duane-Hunt limit”, is given by equation (4a). However, most of the electrons from the cathode will give off only a fraction of their kinetic energy as roentgen radiation during one process of retardation at the anode or will be retarded in several steps. A continuous spectrum of roentgen radiation, composed of roentgen quanta with energies $\leq h\nu_0$, will therefore appear. *The total energy*, I_{tot} , of the continuous roentgen spectrum is approximately proportional to the atomic number Z of the target material and to the square of the potential difference V between the anode and the cathode, provided the electron current in the roentgen tube is constant [91, p. 34]. Thus

$$I_{\text{tot}} = C_1 Z V^2 \quad \dots\dots\dots (6),$$

where C_1 is a constant of proportionality.

The energy distribution in the continuous roentgen spectrum in the wavelength region between 1 kX.U. and 2.8 kX.U. has been investigated by KULENKAMPFF [61]. He determined the relation between the energy distribution and the atomic number Z of the anode material for eight metals (Al,

Fe, Co, Ni, Cu, Ag, Sn, Pt) at a voltage of 10.5 kV across the tube, and the voltage dependence of the energy distribution was investigated for voltages between 7 and 12 kV with platinum and silver as anode material. The original distribution of the intensity in the continuous roentgen spectrum at the atoms of *the massive target* could be represented according to the following equation:

$$I_\nu = C_2[Z(\nu_0 - \nu) + B_1Z^2] \dots\dots\dots (7).$$

I_ν is the intensity of roentgen radiation with the frequency ν , C_2 and B_1 are constants, and ν_0 is the same symbol as in equation (2). In equation (7) the intensity of the continuous roentgen spectrum is given as a function of the frequency of the emitted roentgen radiation, i.e.

$$I_\nu = f(\nu) \dots\dots\dots (7\ a).$$

However, the distribution of the intensity is often represented as a function of the wavelength, i.e.

$$I_\lambda = f(\lambda) \dots\dots\dots (8),$$

where I_λ is the intensity of the roentgen radiation with the wavelength λ . When equations (7 a) and (8) are plotted graphically, the areas $I_\nu \cdot d\nu$ and $I_\lambda \cdot d\lambda$ represent the energy of the roentgen radiation at the frequency ν and at the wavelength λ respectively. If the two equations (7 a) and (8) shall express the energy distribution in the same continuous roentgen spectrum, these two products are equal, but a minus sign must be introduced since an increase of ν corresponds to a decrease of λ according to equation (3). Thus

$$I_\nu d\nu = -I_\lambda d\lambda \dots\dots\dots (9),$$

or

$$\frac{d\nu}{d\lambda} = -\frac{I_\lambda}{I_\nu} \dots\dots\dots (9\ a).$$

Equation (3 a) differentiated with respect to λ gives:

$$\frac{d\nu}{d\lambda} = -\frac{10^8 c}{1.00206 \lambda^2} \dots\dots\dots (10).$$

Combining (9 a) and (10) gives the relation between I_λ and I_ν :

$$I_\lambda = \frac{10^8 c}{1.00206 \lambda^2} I_\nu \dots\dots\dots (11).$$

When equations (7) and (11) are combined the intensity distribution in the continuous roentgen spectrum is obtained as a function of the wavelength, expressed in kX.U.:

$$I_{\lambda} = \frac{CZ}{\lambda^2} \left(\frac{1}{\lambda_0} - \frac{1}{\lambda} \right) + \frac{BZ^2}{\lambda^2} \quad \dots\dots\dots (12),$$

where C and B are constants with $B \ll C$, and λ_0 is the short wavelength limit according to equation (4 a). If equation (12) is differentiated with respect to λ the wavelength $\lambda_{\max}$, corresponding to the maximum value of I_{λ} , is obtained.

$$\frac{dI_{\lambda}}{d\lambda} = \frac{CZ[3\lambda_0 - 2\lambda(1 + BC^{-1}Z\lambda_0)]}{\lambda_0\lambda^4} \quad \dots\dots\dots (13),$$

from which

$$\lambda_{\max} = \frac{3\lambda_0}{2(1 + BC^{-1}Z\lambda_0)} \quad \dots\dots\dots (14).$$

As $B \ll C$, the following expression is valid to a close approximation:

$$\lambda_{\max} = \frac{3\lambda_0}{2} \quad \dots\dots\dots (14 \text{ a}).$$

Neglecting the second term equation (12) is plotted graphically in Fig. 1 (the continuous line).

From theoretical calculations of the distribution of the intensity in the continuous roentgen spectrum KRAMERS [59] has arrived at expressions which agree with the empirical equations for a thick target. In later experiments the same energy distribution in the continuous roentgen spectrum was found at voltages between 20 and 50 kV with a tungsten anode [62]. In a series of papers DE WAARD [101, 102, 103] has derived the energy distribution in a filtered continuous roentgen spectrum for some different forms of the high tension of the roentgen tube.

In the extremely soft roentgen region only a few determinations of the shape of the continuous spectrum from a thick target have been made. Using a Geiger counter spectrometer STEPHENSON and MASON [92] investigated the energy distribution in the continuous roentgen spectrum for voltages between 2.0 and 1.1 kV in the wavelength region from 8 to 14 kX.U. The total integrated energy was found to be proportional to the atomic number of the target material and to the cube of the voltage or to an expression of the form $(V - 0.5)^2$, if V is expressed in kV. The wavelength of maximum

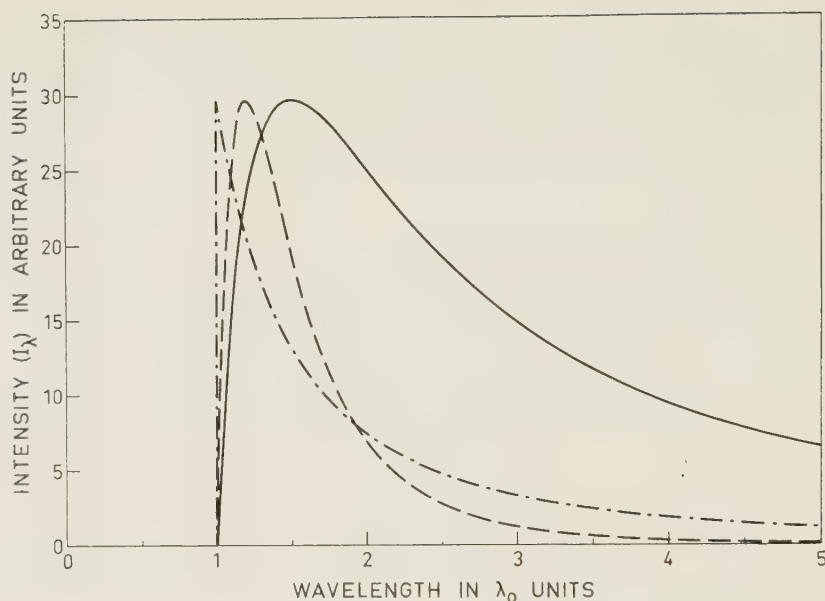


Fig. 1. The distribution of the intensity in the continuous roentgen spectrum as a function of the wavelength. The continuous line, the dash line, and the dash and dotted line correspond to equations (12), (15), and (16) respectively.

intensity, $\lambda_{\max}$, was situated at $1.05 \lambda_0$ to $1.20 \lambda_0$, where λ_0 is the short wavelength limit, as contrasted with the expression for higher voltages in equation (14 a). The empirical expression

$$I_{\lambda} = \frac{C_3 Z}{\lambda^5} \left(\frac{1}{\lambda_0} - \frac{1}{\lambda} \right) \dots\dots\dots (15)$$

satisfied the experimentally determined distributions of the energy in the continuous spectrum between 8 and 12 kX.U. C_3 is a constant of proportionality, and the other symbols are the same as in equation (12). Equation (15) is plotted graphically in Fig. 1 (the dash line).

For voltages from 2.0 to 0.9 kV across the roentgen tube, in the wavelength region between 6 and 20 kX.U., the energy distribution agreed qualitatively with that found at higher voltages [80]. The increase of the energy of the continuous spectrum with Z (the atomic number of the target material) was found to be lower than that corresponding to a direct proportionality to Z .

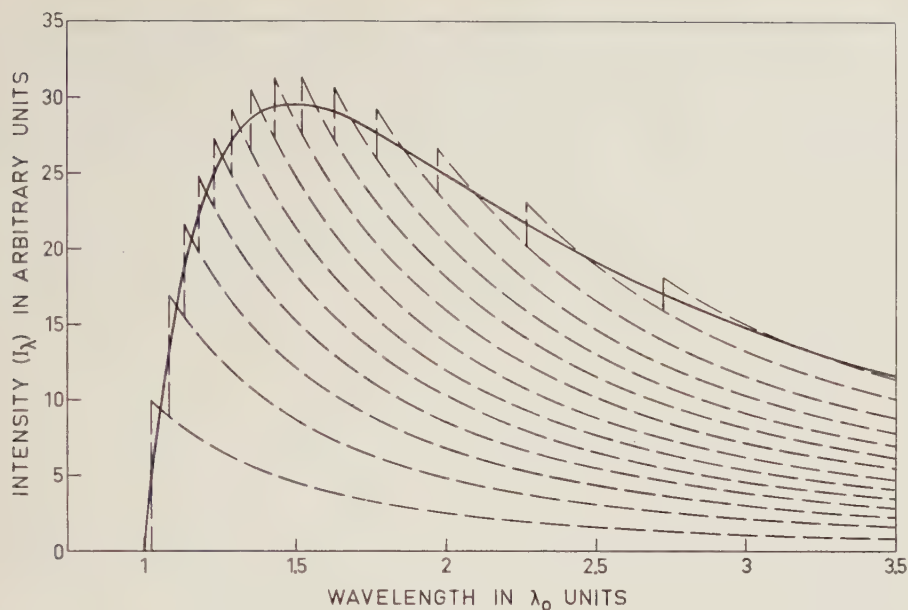


Fig. 2. The continuous spectrum from a massive target (the continuous line) as the sum of a series of thin target spectra (the dash lines).

The energy distribution in the continuous roentgen spectrum from a *thin target* has been calculated theoretically [59] and has also been determined experimentally with thin foils of aluminium and gold as a target [81]. For a thin target I_λ has its maximum at λ_0 and decreases proportionally to λ^{-2} with increasing wavelength according to

$$I_\lambda = \frac{C_4}{\lambda^2} \quad (\lambda \geq \lambda_0) \quad \dots\dots\dots (16),$$

where C_4 is a constant. This function is also represented graphically in Fig. 1 (the dash and dotted line). As only the *relative* distribution of the intensity in the continuous roentgen spectrum is of importance in this discussion, the arbitrary units of the scales on the axis of ordinates have been chosen to make the maximum values of I_λ equal on the three curves according to equations (12), (15), and (16) respectively.

The continuous roentgen spectrum for a thick target can be considered as composed of spectra from a series of thin targets, situated at increasing depths below the surface of the anode. As the energy of the individual electrons incident on the anode diminishes with increasing depth below the

surface of the anode, the series of the thin target spectra will have increasing values of λ_0 according to equations (2) and (3 a). This is shown in the diagram in Fig. 2. *At low voltages* the range of the incident electrons in the anode is small, and the self-absorption of the generated roentgen rays in the target material is strong. Therefore, the continuous spectrum for a massive target at low voltages is composed of relatively few thin target spectra, which means that the distribution of the energy in the continuous spectrum from a massive target then approaches that from a thin target [24]. This can be seen in Fig. 1, where the energy distributions according to equations (15) and (16) have a similar shape.

B. The Characteristic Roentgen Spectra

The incident cathode rays can expel electrons from inner orbits of the atoms in the target, provided the kinetic energy of the incident electrons exceeds certain values. The greatest energy is required to remove a K electron from the innermost orbit, the K orbit, as these electrons are most firmly bound to the nucleus of the atom. The removal of an L, M, N . . . electron from the L, M, N . . . orbit, respectively, requires progressively smaller energies. The L orbit is split in three sublevels, called the L_I , L_{II} , and L_{III} orbits the M orbit in five sublevels, M_I , M_{II} , M_{III} , M_{IV} , and M_V orbits, and the N orbit in seven sublevels. Only the K orbit is not split, and the subdivision of orbits still farther from the nucleus are of no importance for the present work. The energies necessary to expel electrons from the sublevels in an orbit become smaller in sequence from L_I to L_{III} , and from M_I to M_V , though the relative energy differences are rather small within a main orbit.

The vacant space in the inner orbit is rapidly occupied by an electron from an orbit farther from the nucleus of the atom. A roentgen quantum is then emitted, and its energy is given according to the equation

$$h\nu = E_1 - E_2 \dots\dots\dots (17),$$

where h is Planck's constant and ν the frequency of the emitted roentgen quantum. E_1 is the energy (erg) of the excited atom with a vacant space in an inner orbit, and E_2 is the energy of the atom after the electron transition to the inner orbit. The difference $E_1 - E_2$ is an atomic property of the target material, and can for each element take only a limited number of values, depending upon from which inner orbit an electron was expelled by the

incident cathode rays and from which orbit more distant from the nucleus an electron occupied the vacant space. The sequence of values of the difference is characteristic of each element, and the different frequencies of the roentgen radiation according to equation (17) constitute *the characteristic roentgen spectrum* of the anode material. For each element the emission spectrum is divided in different series of frequencies, the K, L, M . . . roentgen spectrum, according as the initial vacant space is situated in the K, L, M . . . orbit, respectively.

The atomic energy level diagram for platinum is shown in Fig. 3, where the O levels are omitted. Along the vertical axis the energies necessary to expel an electron from the different levels are plotted in electron-volts (ev) in a logarithmic scale, and the values are taken from HILL, CHURCH, and MIHELICH [43]. 1 ev corresponds to $1.60207 \cdot 10^{-12}$ ergs [25]. The energies, expressed in ev, have the same numerical values as the minimum excitation voltages for the individual levels, and correspond to the different possible values of E_1 and E_2 for platinum in equation (17), when expressed in ergs. The electron transitions between the levels, diagrammatically represented in Fig. 3, are governed by the quantum numbers of the different levels, and some transitions are not possible. For the individual, possible transitions the probabilities are very different and determine the relative intensities of the emission lines in a certain series of the roentgen spectrum of an element. The energy differences $E_1 - E_2$ of the more frequent electron transitions are shown in Fig. 3. They correspond to the more intense roentgen emission lines of platinum and are marked with the notations of these lines. Along the horizontal, logarithmic wavelength scale in the lower part of the diagram the most intense emission lines are plotted, and the widths of the lines in the diagram are in qualitative agreement with the relative intensities within the K, L, and M series, respectively.

As, for a particular element, the energy differences $E_1 - E_2$ are greatest when the K orbit is concerned, the frequencies of the roentgen emission lines in the K spectrum are highest. The L lines of that element have lower frequencies, and so on. Naturally a particular group of roentgen lines can only appear when the corresponding orbit is an inner orbit in the atom in question. When the kinetic energy of the incident electrons is high enough to eject electrons from a particular level in the atoms of the target material, all the lines in the corresponding series of the characteristic roentgen spectrum are emitted simultaneously. Therefore, the L lines of an element, for instance,

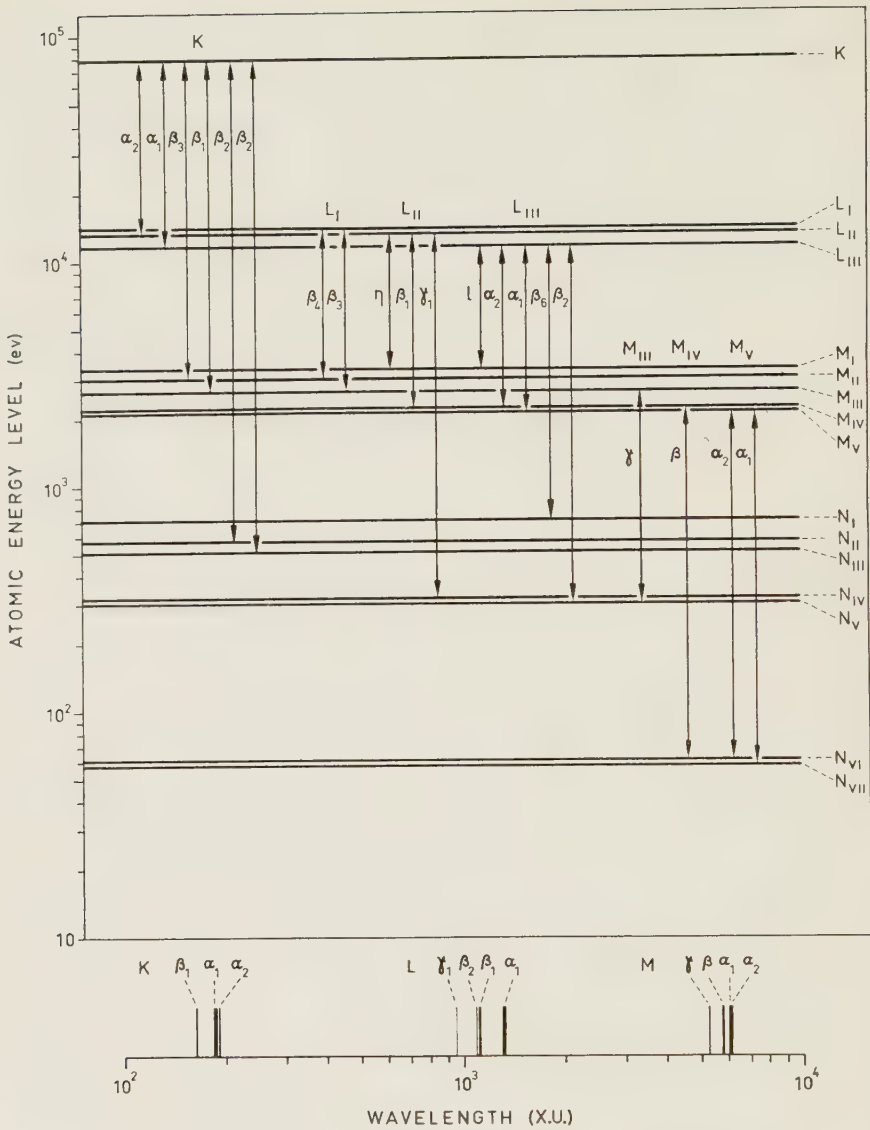


Fig. 3. The atomic energy level diagram of platinum.

can be divided in three groups with somewhat different minimum excitation voltages, corresponding to the three sublevels of the L orbit.

The energy, necessary to eject an electron from a particular orbit to the outside of the atom, is greater the higher the atomic number of the anode

material. The same holds for the difference $E_1 - E_2$ in equation (17). Therefore, the frequency of a characteristic roentgen line, corresponding to a particular electron transition, is higher the higher the atomic number of the element in question. A compilation of wavelengths of roentgen emission lines has been published by CAUCHOIS and HULUBEI [13].

The wavelengths of these roentgen emission lines are characteristic of the anode material in question and practically independent of the chemical combination of the element, particularly when the difference $E_1 - E_2$ in equation (17) is great, i.e. for roentgen lines with relatively high frequencies (short wavelengths). However, the configuration of the valence electrons in the outermost orbit of the excited atoms in different chemical compounds has a small influence upon the absolute values of $E_1 - E_2$. If the difference $E_1 - E_2$ is relatively small, i.e. for long wavelength emission lines in the extremely soft roentgen region, the chemical combination of the target material will have a measurable influence upon the wavelengths of the roentgen spectral lines. The average wavelength of the $K\alpha_1$ line of sulphur is, for instance, 5361.15 X.U. in sulphides (S^{2-}), 5360.83 X.U. in rhombic sulphur (S^0), 5358.63 X.U. in sulphites (S^{4+}), and 5358.08 X.U. in sulphates (S^{6+}) [31].

The intensity of a roentgen emission line as a function of the voltage across the roentgen tube is given by the following empirical expression, provided the minimum excitation potential is exceeded, and the electron current in the roentgen tube is constant:

$$I \approx C_s (V - V_0)^{p_1} \dots \dots \dots (18).$$

I = the intensity of a particular roentgen line.

C_s = a constant of proportionality.

V = the voltage applied to the roentgen tube (kV).

V_0 = the minimum excitation potential (kV) for the roentgen line in question.

From several experimental determinations the average value of the exponent p_1 is about 1.7, which holds up to $V = 4 V_0$ [91, p. 49]. At higher voltages the exponent becomes smaller.

CHAPTER 2

The Absorption, Scattering, and Refraction of Roentgen Rays

A. The General Laws for Absorption

By passage through an element monochromatic roentgen radiation is weakened according to *Lambert's law* of absorption, which has the same form in the roentgen region as it has in the ordinary optical region.

$$I_1 = I_0 e^{-\mu l} \quad \dots\dots\dots (19).$$

I_0 = the intensity of the incident roentgen radiation.

I_1 = the intensity of the transmitted roentgen radiation.

e = the base of the natural logarithms = 2.71828.

μ = the linear absorption coefficient (cm^{-1}).

l = the thickness of the absorbing layer (cm).

The law is valid if the roentgen beam is not too wide or divergent. For a divergent beam the length of the path through the absorbing layer is not the same for all the rays. The first limiting condition depends on the nature of the absorbing process and will be explained later in this chapter.

For roentgen radiation the absorption per unit of mass is usually given instead of the absorption per unit of length, which depends on the physical state of the absorber. If the density of the absorbing element is $\rho \text{ g} \cdot \text{cm}^{-3}$, the product ρl represents the mass, m , of absorbing material per unit of area, expressed in $\text{g} \cdot \text{cm}^{-2}$. Equation (19) then takes the form

$$I_1 = I_0 e^{-\frac{\mu}{\rho} m} \quad \dots\dots\dots (20).$$

where $\frac{\mu}{\rho}$ is the mass absorption coefficient.

As the absorption per gram of the absorbing element is given by the mass absorption coefficient, the absorption per atom of the absorber is obtained from

$$\mu_a = \frac{\mu}{\rho} \cdot \frac{N}{N_0} \quad \dots\dots\dots (21).$$

μ_a = the atomic absorption coefficient.

A = the atomic weight of the absorber.

N_0 = Avogadro's number = $6.0247 \cdot 10^{23}$ mole $^{-1}$ [25].

N_0/A = the number of atoms per gram of the absorber.

The inner electron shells in the atoms of the absorber are of decisive importance for the absorption of roentgen rays. Therefore, μ_a is almost completely independent of the physical and chemical state of the absorbing element, but is a function of the frequency of the incident roentgen radiation, and of the atomic number of the absorber. As N_0/A is constant for each element the mass absorption coefficient has the same characteristics according to equation (21).

The following expression is a close approximation to the composite mass absorption coefficient, $\left(\frac{\mu}{\rho}\right)_{\text{total}}$, for a chemical compound or a mixture composed of n elements with percentages of weight a_i and individual mass absorption coefficients $\left(\frac{\mu}{\rho}\right)_i$.

$$\left(\frac{\mu}{\rho}\right)_{\text{total}} = \sum_{i=1}^n \frac{a_i}{100} \left(\frac{\mu}{\rho}\right)_i \dots\dots\dots (22).$$

If the absorber is composed of n different elements, each with the mass absorption coefficient $\left(\frac{\mu}{\rho}\right)_i$ and the mass m_i g·cm $^{-2}$, the following expression is obtained from equations (20) and (22) :

$$I_1 = I_0 e^{-\sum_{i=1}^n \left(\frac{\mu}{\rho}\right)_i m_i} \dots\dots\dots (23).$$

When roentgen radiation with wavelengths longer than 100 X.U. pass through matter, the true photoelectric absorption and the scattering are the two processes causing the loss of energy, as expressed by the mass absorption coefficient. Therefore

$$\frac{\mu}{\rho} = \frac{\tau}{\rho} + \frac{\sigma}{\rho} \dots\dots\dots (24),$$

where $\frac{\tau}{\rho}$ is the mass photoelectric absorption coefficient and $\frac{\sigma}{\rho}$ is the mass scattering coefficient.

B. The Scattering Process

In the scattering process the roentgen quanta incident on an element remain as photons but deviate from their original path, either with the same frequency as in the incident beam, *coherently scattered radiation*, or with a somewhat lower frequency, *incoherently scattered radiation*, the *Compton scattering*. The coherently scattered radiation is caused by forced oscillations of electrons in the absorbing material, generated by incident photons. The oscillating electrons reemit the radiation in all directions with the same frequency as that of the incident roentgen quanta. At the Compton scattering, electrons in the absorber are recoiled by incident photons, which deviate and give off a part of their energy as kinetic energy of the recoil electrons. Thus these incoherently scattered quanta have lower energy (i.e. lower frequency) than the incident quanta.

The relation between the mass scattering coefficient and *the scattering per electron*, σ_e , is given by

$$\frac{\sigma}{\rho} = \sigma_e N_0 \frac{Z}{A} \quad \dots\dots\dots (25),$$

where Z is the atomic number and A the atomic weight of the scattering substance, and N_0 is Avogadro's number. According to the classical theory, the scattering per electron was independent of the wavelength of the roentgen radiation and of the scattering element. It had the value

$$\sigma_e = \frac{8\pi}{3} \cdot \frac{e_e^4}{m_e^2 c^4} \quad \dots\dots\dots (26),$$

where e_e is the charge and m_e ($=9.1085 \cdot 10^{-28}$ g [25]) the mass of the electron, and c the velocity of light. The numerical value of $\sigma_e N_0$ in equation (25) is then 0.401.

However, experimental determinations of the mass scattering coefficient indicate a wavelength dependence, i.e. $\frac{\sigma}{\rho}$ for an element is smaller at shorter wavelengths. This is in complete agreement with *the Klein-Nishina formula*, derived from quantum mechanics applied to the scattering process [56; 91, p. 89; 15, p. 163]. An accurate testing of the Klein-Nishina formula for roentgen radiation with long wavelengths is impossible, especially for scattering elements with high atomic numbers, since, under these conditions, the values of the mass photoelectric absorption coefficients have a dominating

Table I. $\sigma_e N_0$ for different wavelengths according to the Klein-Nishina formula

Wavelength A.U.	0.1	0.2	0.5	1.0	2.0	5.0	10.0
$\sigma_e N_0$	0.281	0.329	0.367	0.384	0.394	0.398	0.400

influence upon the experimental results. The values of $\sigma_e N_0$ given in Table I have been computed by VICTOREEN [100] from the assumption that the Klein-Nishina formula holds also for roentgen rays with long wavelengths. The difference between these values and the classical value 0.401 is negligible at longer wavelengths. As $Z/A \leq 0.5$ for all elements (except hydrogen), the maximum value of the mass scattering coefficient is about 0.2 according to equation (25).

C. The Photoelectric Absorption Process

In the photoelectric absorption process incident roentgen quanta eject electrons from inner orbits of the atoms in the absorber in the same way as cathode rays expel electrons from the anode material. Thus the energy of a photoelectrically absorbed roentgen quantum is transformed to potential energy of the excited atom and to kinetic energy of the ejected electron, *the photoelectron*. These electrons give off their kinetic energy by ionizing other atoms in the absorbing layer. When the atoms return to their unexcited state, the potential energy reappears as roentgen radiation, characteristic of the excited atoms of the absorber, and emitted in all directions. The frequency of this characteristic radiation is given by equation (17).

The number of atoms in a particular excited state is always greater than the corresponding number of emitted roentgen quanta, owing to *the Auger effect*. This depends on the fact that some of the photons, emitted from the excited atoms, are photoelectrically absorbed in outer orbits in the same atoms, thus expelling secondary photoelectrons, called *Auger electrons*.

The fluorescence yield for the K orbit of an element, w_K , is defined as the ratio between the number of emitted K roentgen quanta and the number of K electrons, expelled by photoelectric absorption in the K orbit. The fluorescence

Table II. The fluorescence yield for different electron orbits for some elements

Element	w_K	w_L	w_M	Reference
6 C	0.0009			[19]
12 Mg	0.013			[41]
24 Cr	0.263			[41]
29 Cu	0.401			[2]
42 Mo	0.724			[2]
42 Mo		0.067		[71]
47 Ag	0.795			[2]
47 Ag		0.10		[71]
78 Pt		0.348		[71]
92 U		0.45	0.06	[71]

yield for a particular orbit increases with the atomic number of the element in question. For each element w_K is greater than w_L , and so on. In Table II some experimental determinations of the fluorescence yield have been compiled from the physical literature.

With the exception of the critical absorption limits, which will be discussed in the next section, the mass photoelectric absorption coefficient is approximately proportional to the third power of the wavelength of the incident roentgen radiation and to the fourth power of the atomic number of the absorbing element, whereas the mass scattering coefficient is approximately constant at wavelengths longer than 2 kX.U. At wavelengths longer than about 0.2 kX.U. the photoelectric absorption dominates over the "false" absorption due to scattering, at first for elements with high atomic numbers, and with increasing wavelengths also for elements with low atomic numbers. Therefore, *the mass scattering coefficient can be totally neglected in comparison with the mass photoelectric absorption coefficient for roentgen rays with wavelengths longer than 2.5 kX.U.*, which is the important wavelength region when roentgen absorption spectrophotometry is applied in quantitative cytochemistry. For these soft roentgen rays the mass photoelectric absorption coefficients are numerically equal to the corresponding mass absorption coefficients, as the values of the mass scattering coefficients are generally much smaller than the errors of the experimental determinations of the mass absorption coefficients. Hydrogen is the only element of biological interest for which this is only approximately correct up to about 8 kX.U., but this

has no influence upon the cytochemical applications of roentgen absorption spectrophotometry.

As the secondary characteristic roentgen emission spectrum, i.e. the fluorescence radiation, and the scattered radiation are emitted in all directions from their points of origin in the absorbing layer, a small fraction of these secondary roentgen rays is always added to the transmitted roentgen beam. The increase of the intensity is negligible for a narrow beam, but it may have a measurable influence upon the absorption values if the beam is too broad, which explains the first limitation of the Lambert law.

D. The Absorption Edges

In the roentgen region the elements do not selectively absorb incident radiation with wavelengths corresponding to their characteristic emission lines. For each element the mass photoelectric absorption coefficient is a continuously increasing function of the wavelength of the incident roentgen radiation, with the exception of specific discontinuities at certain wavelengths. These *critical absorption limits or edges* are related to the different atomic energy levels of the absorbing element.

Incident roentgen quanta with wavelengths shorter than that corresponding to an absorption edge of the absorbing element have enough energy to eject electrons from a particular orbit in the atoms of the absorber, i.e. these quanta can be photoelectrically absorbed in the orbit in question. If, however, the incident roentgen quanta have wavelengths longer than that corresponding to the absorption limit, their energy is insufficient to expel electrons from that particular level, i.e. no photoelectric absorption can occur in this orbit. Therefore, in the immediate proximity of an absorption edge, the total photoelectric absorption in the different orbits of the atoms of the absorbing element, as expressed by the mass photoelectric absorption coefficient, is greater on the short wavelength side of the limit than on the long wavelength side, as, on the latter side, no photoelectric absorption occurs in the orbit corresponding to the absorption limit.

Roentgen quanta with the frequency ν_{cr} , corresponding to the wavelength of a critical absorption limit of an element, have the minimum energy necessary to eject photoelectrons from the corresponding level in the atoms of the absorbing element. The potential energy of each of these excited atoms

is then equal to the energy $h\nu_{cr}$ of such a roentgen quantum. As, for each element, this potential energy is greater than the energies of the corresponding characteristic roentgen emission quanta, all the characteristic roentgen emission lines, related to a particular atomic energy level, have wavelengths longer than that of the corresponding critical absorption limit.

The notation of an absorption edge is the same as that of the corresponding electron orbit. *The wavelengths of the absorption limits are characteristic of the absorbing element and practically independent of the physical and chemical state of the absorber.* The absorption limits are displaced towards shorter wavelengths, when the atomic number of the absorbing element increases. Values of critical absorption wavelengths have been compiled by COMPTON and ALLISON [16, pp. 792—794] and by CAUCHOIS and HULUBEI [13]. The wavelengths can also be calculated from the values of the atomic energy levels of the absorbing element [43].

In Fig. 4 the values of the mass absorption coefficient of platinum [16, pp. 800—806; 46] are plotted as a function of the wavelength in a double logarithmic scale. Only the K, L, and M absorption edges have been plotted, since the values of the mass absorption coefficient and the magnitude of the absorption edges of platinum are not accurately known at longer wavelengths. At wavelengths longer than about 0.5 kX.U. the mass scattering coefficient of platinum can be neglected in comparison with the mass photoelectric absorption coefficient.

The general shape of the absorption spectrum of an element, as shown in Fig. 4, is found when the resolution along the wavelength scale is low. If the absorption spectrum for a solid absorber is determined with a high resolution, *a series of absorption maxima and minima is found on the short wavelength side of the absorption edges*, and the physical and chemical state of the absorbing element has a small influence upon the position of the main edges.

If the energy of the incident roentgen quanta is only slightly higher than the energy necessary to expel an electron from a particular level, the ejected photoelectron has a small kinetic energy. There is evidence that such an electron can have only certain discrete energies to be able to move in a particular direction in the crystal lattice of the solid absorber, and only those incident roentgen quanta are absorbed, which eject photoelectrons with such energies [60]. As the photoelectrons move in all directions, the integrated effect of the crystal lattice will be a series of fluctuations in the absorption

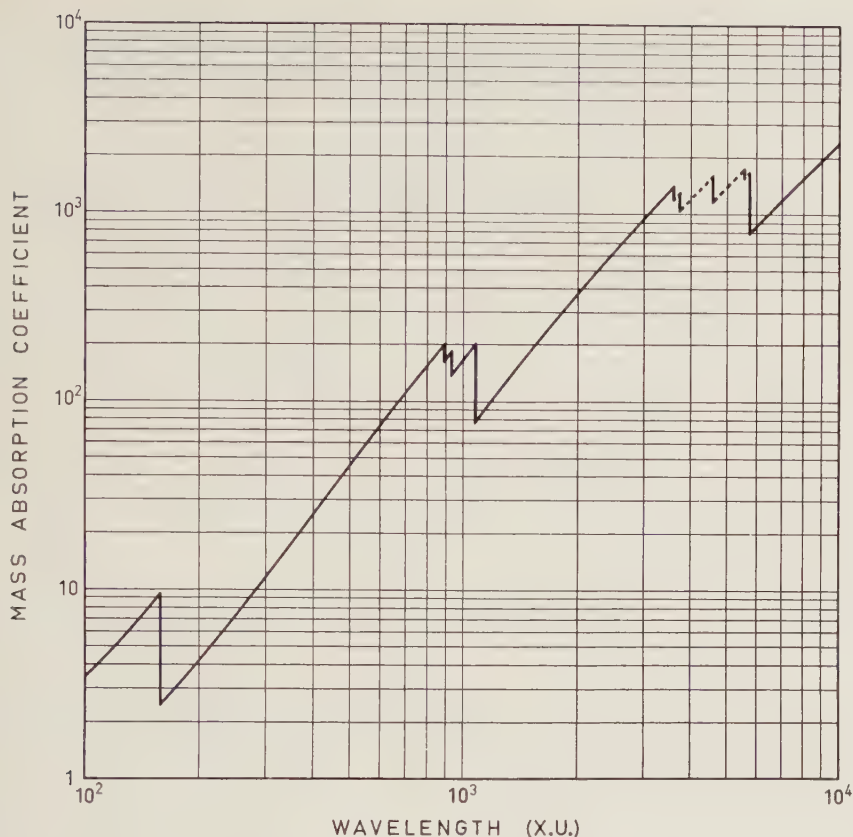


Fig. 4. The mass absorption coefficient of platinum as a function of the wavelength of the incident roentgen radiation.

spectrum on the short wavelength side of the absorption edge. The extension and the shape of this *fine structure* show a great variation. The positions of the absorption maxima and minima are usually expressed as energy differences (ev) in relation to the energy corresponding to the main edge.

The atoms of the absorbing element and other kinds of atoms in chemical compounds have also an influence upon the fine structure. This is exemplified in an investigation of the fine structure at the K absorption edge of iron and of its oxides [17, 18]. At this edge the fine structure is mainly an atomic property in a region up to 70 ev from the main edge. It is determined by the surrounding atoms in a region from 70 to 175 ev and depends on the crystal lattice in a region above 175 ev. For a discussion of the fine structure

Table III. The wavelength of the K absorption edge of sulphur in different chemical compounds according to LINDH [73, p. 291]

Compound	Oxidation step	Wavelength X.U.
Cr_2S_3	S^{2-}	5011.7
ZnS	S^{2-}	5005.3
Monoclinic S	S^0	5009.0
Rhombic S	S^0	5008.6
BaSO_3	S^{4+}	4996.4
CuSO_3	S^{4+}	4995.6
K_2SO_4	S^{6+}	4988.4
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	S^{6+}	4987.3

reference should be made to COMPTON and ALLISON [16, pp. 662—671] and to SKINNER [90].

A line absorption structure has been found on the long wavelength side of the K absorption edge for some metal oxides, which has been discussed by SANNER [85].

The influence of chemical combination upon the position of the absorption edges has been extensively investigated [73, pp. 281—314; 88, pp. 278—294]. It has been found that the wavelength of the main K absorption edge depends on the oxidation step of the absorbing element. *The limit is displaced towards shorter wavelengths when the oxidation step of the absorber increases*, but other kinds of atoms in chemical compounds of the element in question have also a small influence upon the position of the absorption edge. As the effect of chemical combination upon the wavelength of roentgen emission lines was exemplified by the $\text{K}\alpha_1$ line of sulphur, the wavelength of the K absorption edge of sulphur in some different compounds are given in Table III.

As will be discussed in more detail in connection with the cytochemical applications of roentgen absorption spectrophotometry, the influence of chemical combination upon the position of the absorption edges, the fine structure on the short wavelength side of the absorption limits, and the absorption lines, can influence upon roentgen absorption measurements on biological material only in exceptional cases [29]. The relative magnitudes

of the absorption edges are discussed in the next section in connection with the numerical values of the mass absorption coefficients.

E. Empirical Absorption Formulae and the Magnitude of the Absorption Edges

The mass photoelectric absorption coefficient is a function of the wavelength λ of the incident roentgen radiation and of the atomic number Z of the absorbing element. Empirical formulae have generally been of the type

$$\frac{\tau}{\rho} = k\lambda^u Z^v \quad \dots\dots\dots (27),$$

where k , u , and v are constants. In all absorption formulae in this section λ is expressed in kX.U. However, with fixed constants, the validity of such expressions is restricted to only one side of a critical absorption edge, and to a limited range for λ and Z , as the exponents u and v , too, are functions of λ and Z . For the older expressions of this type reference should be made to WALTER [104].

In most of the formulae u has a value about 3 and v a value about 4, but in the extremely soft roentgen region these values are changed. From absorption measurements in thin celluloid foils, utilizing extremely soft, continuous, filtered roentgen radiation, HOLWECK [49] found that the mass absorption coefficients for oxygen, nitrogen, and hydrogen were proportional to $\lambda^{2.5}$ in the wavelength range between 44 and 88 kX.U. The atomic absorption coefficients, μ_a , were proportional to $Z^{4.4 \pm 0.4}$ in an investigation of the absorption of the $K\alpha$ line of carbon ($\lambda \approx 44.5$ kX.U.) in carbon, nitrogen, and oxygen [63]. In later absorption measurements with the same radiation, μ_a of carbon, nitrogen, oxygen, and the inert gases were proportional to $Z^{4.4}$ [22, 23].

The magnitude δ_K of a K absorption jump is defined as the ratio between the photoelectric absorption coefficients on both sides and in the immediate proximity of the K absorption limit. The corresponding definition holds for δ_{L_I} , $\delta_{L_{II}}$, $\delta_{L_{III}}$, δ_{M_I} , and so on. The product of the magnitudes of the three absorption jumps in the L group is defined as the *total L absorption jump*, δ_L [16, pp. 530—531].

$$\delta_L = \delta_{L_I} \cdot \delta_{L_{II}} \cdot \delta_{L_{III}} \quad \dots\dots\dots (28).$$

The corresponding expression holds for the total M absorption jump, δ_M .

On an empirical basis JÖNSSON [52] found that the relation

$$\delta_K = \frac{E_K}{E_{L_I}} = \frac{h\nu_K}{h\nu_{L_I}} = \frac{\lambda_{L_I}}{\lambda_K} \dots\dots\dots (29)$$

was consistent with the experimental values of δ_K . E_K is the excitation energy of the K orbit, and ν_K and λ_K are the frequency and wavelength, respectively, corresponding to the K absorption limit. E_{L_I} , ν_{L_I} , and λ_{L_I} are related to the L_I orbit and the L_I absorption edge in the same way. For δ_L , the total L absorption jump, a similar relation was suggested:

$$\delta_L = \frac{E_{L_I}}{E_{M_I}} \dots\dots\dots (29 \text{ a}).$$

JÖNSSON [52] defined a new photoelectric absorption coefficient, τ_e , the *electronic true absorption coefficient*, which is equivalent with σ_e , the scattering per electron.

$$\tau_e = \frac{\tau}{\rho} \cdot \frac{A}{N_0 Z} \dots\dots\dots (30),$$

where A , Z , and N_0 are the same symbols as in equation (25). For every absorbing element a smooth absorption curve without discontinuities was obtained, when the values of τ_e between the K and L_I absorption edges were multiplied by the corresponding jump factor $\frac{E_K}{E_{L_I}}$, the values of τ_e between

the L_{III} and M_I absorption edges by $\frac{E_K}{E_{M_I}}$, and so on. The electronic true absorption coefficients along the K absorption branch, and the ones transposed to the continuous extension of this branch beyond the K absorption edge, were denoted as $(\tau_e)_K$. *When $\log (\tau_e)_K$ was plotted as a function of $\log Z\lambda$ the curves for different absorbing elements were practically identical.* The difference between the graphical mean and the individual experimentally determined absorption values was in general smaller than ± 5 per cent but in exceptional cases up to ± 15 per cent.

The curve describing $\log (\tau_e)_K$ as a function of $\log Z\lambda$ has a continuously decreasing derivative for increasing values of the product $Z\lambda$. The corresponding equation can therefore be written as

$$(\tau_e)_K = k_1 (Z\lambda)^{f(Z\lambda)} \dots\dots\dots (31),$$

where k_1 is a constant and $f(Z\lambda)$ decreases continuously from 3.0 to 2.3 when $Z\lambda$ increases from 8 to 800. The equation was considered to be valid up to a wavelength of 12 kX.U. Numerical values of $(\tau_e)_{\text{K}}N_0$ for $8 < Z\lambda < 800$ have been published [52, 88, pp. 470—471]. When $(\tau_e)_{\text{K}}$ has been transposed to its original branch on the absorption curve by division by the corresponding jump factor, the mass photoelectric absorption coefficient can be calculated according to equation (30).

Systematic deviations from equation (31) have been found for some elements with low atomic numbers in the wavelength region from 2 to 10 kX.U. [106] and for aluminium at wavelengths between 7 and 25 kX.U. [3]. The mass photoelectric absorption coefficients for seven elements in the wavelength range from 1.66 to 9.87 kX.U., determined by BIERMANN [5], were in good agreement with the values calculated from Jönsson's tables at about 2.5 kX.U., but at longer wavelengths the experimental values were increasingly higher than those calculated. From absorption measurements in the wavelength region between 1.5 and 8.3 kX.U. ANDREWS [1] found that, according to equation (31), the electronic true absorption coefficients could be represented by a smooth curve, which, however, had a significantly higher position as compared with Jönsson's curve at greater values of the product $Z\lambda$.

Some later determinations of the relative magnitudes of the K absorption jump have been consistent with equation (29), i.e. δ_{K} of argon, chlorine, and sulphur [106], and δ_{K} of iron, nickel, copper, zinc, palladium, and silver [77]. However, in other investigations, the expression (29) was found to be approximately correct for manganese ($Z=25$) but gave increasingly too great values for elements with higher atomic numbers [39, 69]. RINDFLEISCH [84] found that δ_{K} could be expressed as a function of the critical absorption wavelength λ_{K} (kX.U.), or of the atomic number Z of the absorbing element, according to

$$\delta_{\text{K}} = 7.2053 \lambda_{\text{K}}^{0.2843} = 63.868 Z^{-0.6207} \dots\dots\dots (32).$$

This expression has been verified in other investigations [79, 42, 70].

In a series of experiments δ_{L_I} and $\delta_{\text{L}_{II}}$ were found to be independent of the atomic number of the absorbing element down to selenium ($Z=34$) [67, 68, 69, 5, 86].

$$\delta_{\text{L}_I} = 1.154 \pm 0.024 \dots\dots\dots (33).$$

$$\delta_{\text{L}_{II}} = 1.373 \pm 0.043 \dots\dots\dots (33 \text{ a}).$$

However, with increasing atomic number of the absorbing element, $\delta_{L_{III}}$ and the relative magnitude of the total L absorption jump, δ_L , decreased continuously from 4.46 and 6.74 respectively for selenium [5] to 2.39 and 3.79 respectively for bismuth ($Z = 83$) [86]. Values of δ_L , calculated according to equation (29 a), are somewhat higher all through, and deviations from the constant values of δ_{L_I} and $\delta_{L_{II}}$ have also been reported [107].

From compilations of available data of the absorption of roentgen radiation in different elements, VICTOREEN [96, 97, 98, 99, 100] has evolved a series of empirical expressions for the calculation of mass absorption coefficients. From about 0.01 kX.U. to the wavelength λ_K at the K absorption limit of the absorbing element the following expression was given:

$$\frac{\mu}{\rho} = C_K \lambda^3 - D_K \lambda^4 + \sigma_e N_0 \frac{Z}{A} \quad \dots \dots \dots (34).$$

For each element C_K and D_K are constants, and λ is the wavelength of the incident roentgen radiation. The last term is the mass scattering coefficient which has been discussed in a previous section in this chapter. If C_K is changed to C_{K-L} or C_{L-M} and D_K to D_{K-L} or D_{L-M} , expression (34) is valid for mass absorption coefficients at wavelengths between the critical absorption limits. On an empirical basis it was found that the constants C_x and D_x could be accurately calculated according to the equations

$$C_x = \frac{1}{\lambda_1 \lambda_2} \cdot \frac{3\pi c_e^2}{m_e c^2} \cdot \frac{N_0}{A} \quad \dots \dots \dots (35)$$

and

$$D_x = \frac{1}{\lambda_1 \lambda_2 \lambda_3} \cdot \frac{3\pi c_e^2}{m_e c^2} \cdot \frac{N_0}{A} \quad \dots \dots \dots (35 \text{ a}).$$

c_e and m_e are the charge and mass of an electron, c the velocity of light, N_0 Avogadro's number, and A the atomic weight of the absorber. λ_1 , λ_2 , and λ_3 are critical wavelengths, calculated from the formula of spectral emission by introducing suitable quantum numbers. Values of C_K and D_K for all elements according to expressions (35) and (35 a), and mass absorption coefficients for all common elements according to equation (34) have been published together with a complete review of the calculations [98, 99, 100].

TELLEZ-PLASENCIA [94] has discussed different empirical expressions for the relative magnitude δ of the absorption discontinuities and has evolved new expressions, which, solved for δ , are as follows:

$$\delta_K = (0.051167 + 0.002482 Z)^{-1} \quad \dots \dots \dots (36),$$

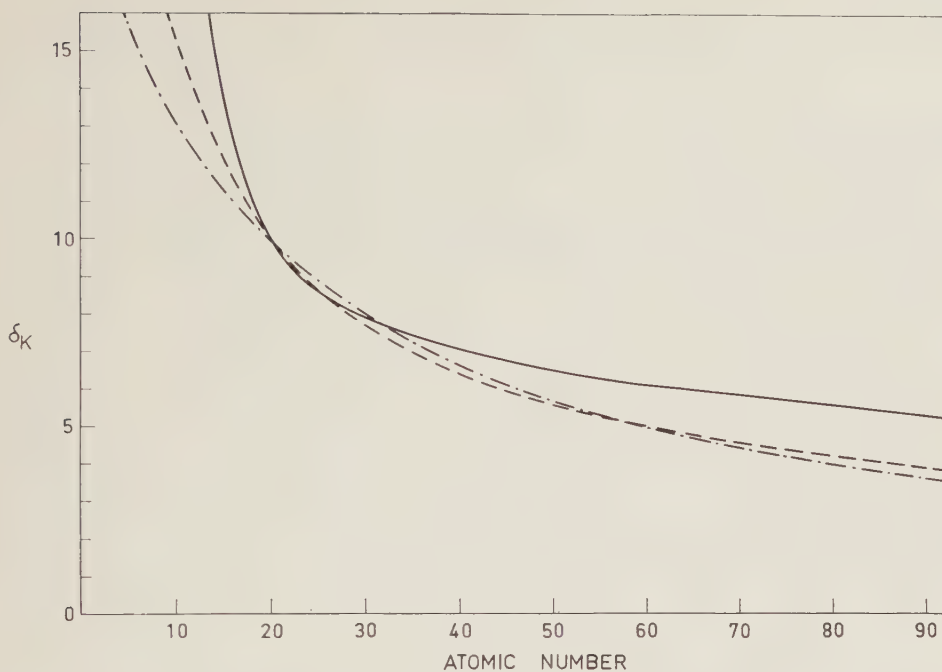


Fig. 5. The magnitude of the K absorption jump δ_K as a function of the atomic number Z of the absorbing element according to equation (29) (the continuous line), equation (32) (the dash line), and equation (36) (the dash and dotted line).

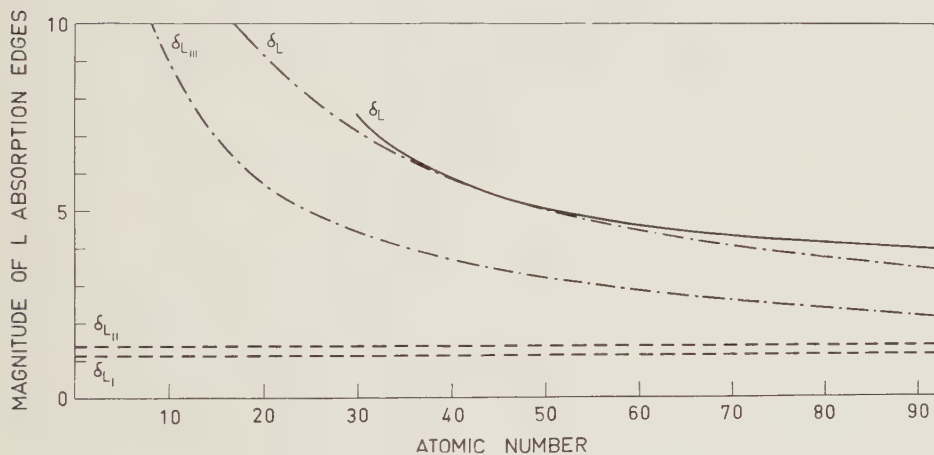


Fig. 6. The magnitudes of the different L absorption jumps, δ_{L_I} , $\delta_{L_{II}}$, and $\delta_{L_{III}}$, and of the total L absorption jump, δ_L , as functions of the atomic number Z of the absorbing element. δ_L is calculated according to equation (29 a) (the continuous line), and equation (37 a) (the dash and dotted line).

$$\delta_{L_{III}} = 38.22 Z^{-0.6332} \dots\dots\dots (37),$$

$$\delta_L = 60.56 Z^{-0.6332} \dots\dots\dots (37 a).$$

For δ_{L_I} and $\delta_{L_{II}}$ expressions (33) and (33 a) were considered to be correct.

In Fig. 5 the relative magnitude of the K absorption jump is plotted as a function of the atomic number of the absorbing element according to the different empirical expressions, given in equations (29), (32), and (36). In Fig. 6 corresponding graphs are plotted of δ_{L_I} and $\delta_{L_{II}}$ according to equations (33) and (33 a), of $\delta_{L_{III}}$ according to equation (37), and of the total L absorption jump according to equations (29 a) and (37 a).

As compared with the determinations of the wavelengths of roentgen emission lines and of absorption edges, the methods of determining absorption coefficients of roentgen radiation have a much lower accuracy, which explains the divergent values of the absorption coefficients in the physical literature. In connection with the cytochemical applications of roentgen absorption spectrophotometry the influence of this relatively low accuracy will be discussed.

F. The Refraction of Roentgen Rays

For roentgen radiation the refractive index μ in various materials is less than 1 by a very small quantity according to the following equation, where δ is obtained from the Drude-Lorentz dispersion formula, applied to electromagnetic radiation with a frequency much higher than that of the electronic resonant frequencies [91, pp. 97—98].

$$1 - \mu = \delta = \frac{e_e^2 \lambda^2}{2\pi m_e c^2} \cdot \frac{N_0 \varrho Z}{A} \dots\dots\dots (38).$$

e_e and m_e are electronic charge and mass, c the velocity of light, and λ the wavelength of the roentgen radiation. N_0 is Avogadro's number, and ϱ , Z , and A are the density ($\text{g} \cdot \text{cm}^{-3}$), the atomic number, and the atomic weight, respectively, of the element in question. $N_0 \varrho Z A^{-1}$ is the number of electrons per cm^3 of the element. For chemical compounds the number of electrons per molecule is substituted for Z and the molecular weight for A . Introducing the numerical values of the constants, expression (38) takes the following form:

$$\frac{\delta}{\lambda^2} = 2.702 \varrho Z A^{-1} \cdot 10^{-6} \dots\dots\dots (38 a),$$

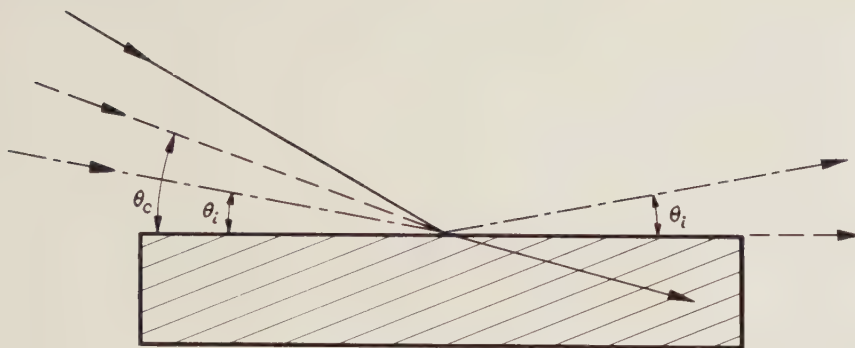


Fig. 7. The total reflection of roentgen rays.

where λ is expressed in Å.U. This expression shows the constant value of $\frac{\delta}{\lambda^2}$ for a given material. In unaltered form, however, equation (38) is not valid at wavelengths in the vicinity of the absorption edges of the material, where the electronic resonant frequencies are of the same magnitude as the frequency of the roentgen radiation [88, pp. 36—42].

As the index of refraction is less than 1, roentgen rays, incident on a surface of a solid or a liquid, are totally reflected, provided the angle of incidence exceeds a certain value. The angle between the incident roentgen rays and the surface, i.e. the incident glancing angle θ_i , must then be smaller than a certain angle of critical grazing incidence θ_c . This is shown in Fig. 7, where the dash and dotted line represents a totally reflected roentgen ray, the dash line a ray with critical grazing incidence, and the continuous line a ray which can only be refracted. As the critical grazing angle is small, the following relation between θ_c and δ can be deduced [91, p. 101]:

$$\theta_c = \sqrt{2\delta} \quad \dots\dots\dots (39),$$

where θ_c is expressed in radians. If expression (38 a) is substituted for δ , the following equation is obtained:

$$\theta_c = 10^{-3}\lambda \sqrt{5.404 \rho Z A^{-1}} \quad \dots\dots\dots (39 a).$$

Thus, for a given material, there is a direct proportionality between the critical grazing angle and the wavelength of the incident roentgen radiation.

The dash curve in Fig. 8 illustrates schematically the relative reflected intensity as a function of the relative glancing angle of the incident monochromatic roentgen radiation for a non-absorbing material. The relative

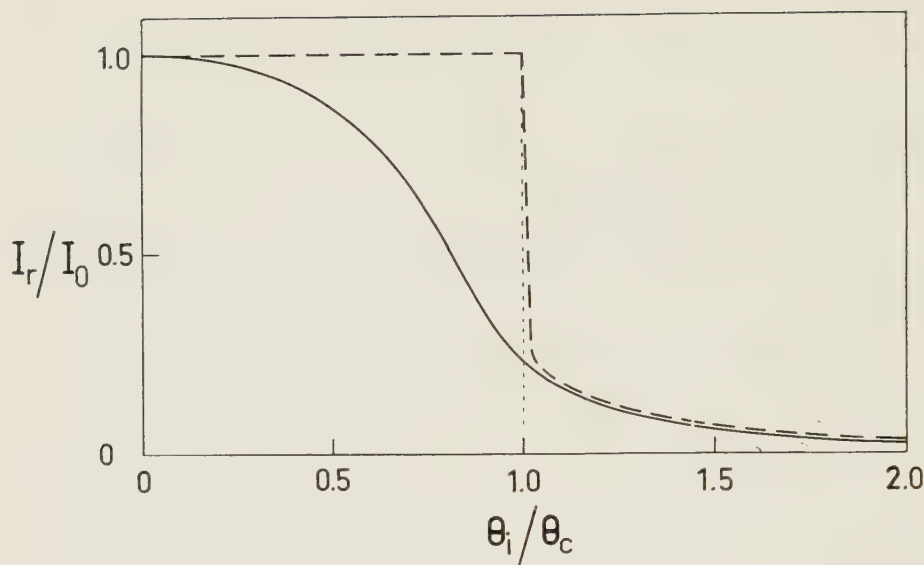


Fig. 8. Schematic diagram of the relative intensity of the reflected roentgen radiation as a function of the relative glancing angle. The dash curve corresponds to a non-absorbing, reflecting material, and the continuous curve illustrates the relative reflected intensity for an absorbing material.

intensity of the reflected roentgen beam is given by the ratio between the intensity of the reflected radiation, I_r , and of the incident radiation, I_0 , and the relative glancing angle is expressed as the ratio between the incident glancing angle θ_i and the critical grazing angle θ_c . The abrupt decrease of the reflected intensity at the critical grazing angle is only found, if the absorption of the roentgen radiation in the reflecting material as negligible. In most cases, however, especially for soft roentgen rays, the absorption cannot be neglected. A complex value of the refractive index must then be introduced, where the complex term is related to the absorption [88, p. 34]. In this case the decrease of the reflected intensity with increasing values of the incident glancing angle is more gradual, which is schematically illustrated by the continuous curve in Fig. 8.

CHAPTER 3

The Production of Monochromatic Roentgen Rays

The roentgen radiation generated in a roentgen tube is always composed of polychromatic roentgen rays, i.e. the continuous roentgen spectrum, and roentgen emission lines characteristic of the target material, which has been discussed in Chapter 1. Different methods have been utilized to obtain monochromatic roentgen radiation from these primary roentgen rays. A short review will be given of the different procedures, which can be of importance for roentgen absorption spectrophotometry applied in quantitative cytochemistry.

A. Methods Based upon the Filtration of the Roentgen Beam

Fig. 9 illustrates schematically the distribution of the intensity in a continuous roentgen spectrum, transmitted through *a filter with no critical absorption limit in the wavelength region in question*. As, in this case, the mass photoelectric absorption coefficient is approximately proportional to the third power of the wavelength, the transmitted roentgen spectrum comprises almost exclusively the short wavelength part of the original continuous spectrum, provided the absorption in the filter is strong.

The method of balanced filters according to Ross is illustrated in Fig. 10. Two elements with adjacent atomic numbers are used alternately in the form of thin foils to filter a continuous roentgen spectrum, and the thicknesses of the foils are chosen to make the distributions of the intensity in the transmitted spectra equal with the exception of the wavelength region between the K absorption edges of the two elements. The difference between the intensities of the two filtered roentgen spectra in this wavelength region, corresponding to the dash area in Fig. 10, causes the divergence between the effects of the two spectra. For a complete discussion of this method reference should be made to KIRKPATRICK [54, 55].

"The filter difference method" according to Küstner is the third method in this group to be discussed. Secondarily excited K emission lines are utilized in this method, which is schematically illustrated in Fig. 11 a—d. A con-

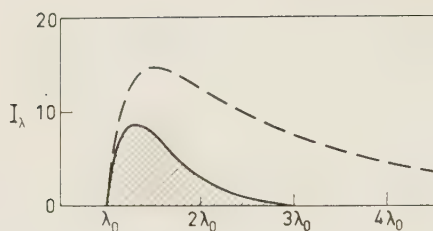


Fig. 9.

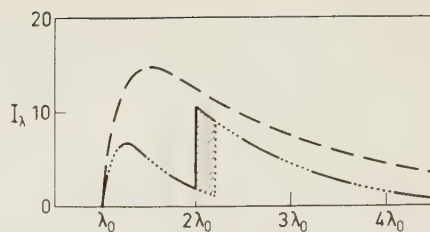


Fig. 10.

Fig. 9. The distribution of the intensity in the continuous roentgen spectrum before and after filtration (the dash curve and the continuous curve, respectively). The dash area corresponds to the total transmitted intensity.

Fig. 10. The method of balanced filters according to Ross.

tinuous roentgen spectrum with the intensity I is incident upon an element Z after passage through a filter in the position A_1 (Fig. 11 a). At B the secondary roentgen radiation is composed of scattered roentgen radiation with the intensity I_s and characteristic roentgen emission lines, mainly the K spectrum, with the intensity I_{e1} . The distribution of the intensity in this secondary spectrum is shown in Fig. 11 b. The filter is then moved to the position A_2 , illustrated in Fig. 11 c. When, in this case, the continuous roentgen spectrum is incident upon Z , the distribution of the intensity in the secondary roentgen spectrum at B is shown in Fig. 11 d. The basic principle of this method is the fact that the scattered radiation I_s , corresponding to the dash area in Fig. 11 b and Fig. 11 d, is identical for the two positions A_1 and A_2 of the filter. However, $I_{e2} \ll I_{e1}$, as the filter is chosen to transmit only about 1 per cent of the intensity of the characteristic roentgen radiation. Therefore, the divergence between the effects of the secondary radiation in the two cases is caused by a characteristic roentgen spectrum with the intensity $I_{e1} - I_{e2}$. For a complete description of this method, including a discussion of the possibilities to eliminate the effect of the $K\beta$ and $K\gamma$ lines by suitable filters or by a correction factor at the calculations, reference should be made to KÜSTNER [64, 65, 66].

The possibility to obtain relatively monochromatic roentgen radiation *with a high intensity* is the main advantage of these methods. However, the method of balanced filters can only be used in the short wavelength region, where the difference between the wavelengths of the K absorption edges of adjacent elements is small, and the possibilities to obtain monochromatic roentgen radiation according to Küstner's method is restricted to some scattered wave-

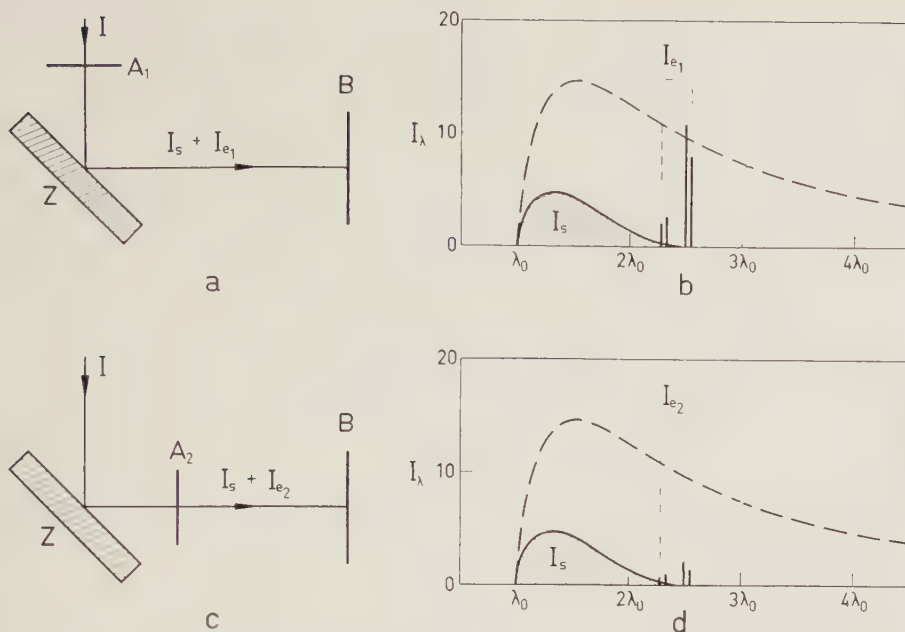


Fig. 11 a—d. "The filter difference method" according to Kuestner.

lengths in the soft roentgen region. When utilizing roentgen absorption spectrophotometry in quantitative cytochemistry, a more general method to obtain monochromatic roentgen radiation is to be preferred.

B. The Diffraction of Roentgen Rays by Crystals; Bragg's Law

In the wavelength region below 20 kX.U. monochromatic roentgen radiation is usually produced by the diffraction of roentgen rays by crystals. When parallel, polychromatic roentgen radiation, incident at certain angles upon a crystal, is scattered by the regularly spaced atoms, the scattered roentgen rays from different parallel atomic planes are reinforced in certain directions by constructive interference. For this case the relation between the wavelength λ of the diffracted roentgen rays, the glancing angle φ , and the grating space of the crystal d , i.e. the distance between the parallel atomic planes (Fig. 12), is given by *Bragg's Law*:

$$n\lambda = 2d \sin \varphi \quad \dots \dots \dots (40).$$

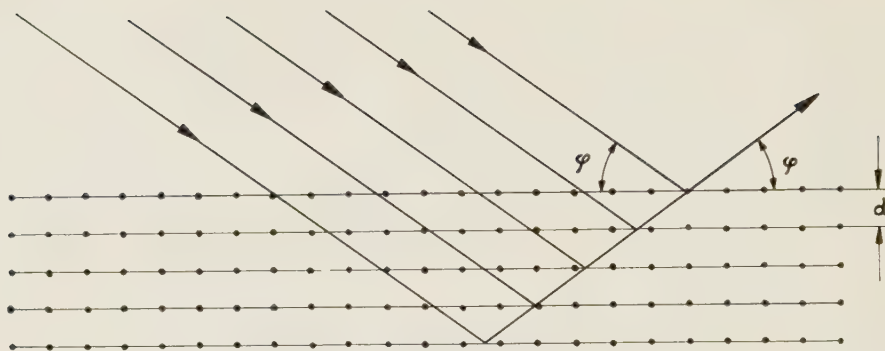


Fig. 12. The diffraction of roentgen rays by a crystal. The rows of points correspond to atomic planes.

d and λ are expressed in X.U., and n is the order of diffraction. If λ_1 is the wavelength corresponding to $n=1$, the wavelengths $\frac{\lambda_1}{2}, \frac{\lambda_1}{3}, \frac{\lambda_1}{4} \dots$ are reinforced simultaneously in the same direction. *Monochromatic roentgen radiation is obtained, if the voltage across the roentgen tube is lower than the excitation voltage for a continuous roentgen spectrum with the short wavelength limit $\frac{\lambda_1}{2}$.* Then, according to equations (2), (3), and (4 a), roentgen quanta with the wavelengths $\frac{\lambda_1}{2}, \frac{\lambda_1}{3}, \frac{\lambda_1}{4} \dots$ have too high quantum energies to be generated at the voltage in question.

When correcting for the refraction of the roentgen radiation in the crystal, Bragg's law takes the following modified form [88, pp. 20—21]:

$$n\lambda = 2d_n \sin \varphi \dots \dots \dots (40 \text{ a}),$$

where d_n is the effective grating space of the crystal at diffraction in the n th order. It is given by the following expression:

$$d_n = d \left(1 - \frac{\delta}{\sin^2 \varphi} \right) = d \left(1 - \frac{4d^2}{n^2} \cdot \frac{\delta}{\lambda^2} \right) \dots \dots \dots (41),$$

where d is the real grating space of the crystal, and $\frac{\delta}{\lambda^2}$ is calculated according to equation (38 a). Values of the constants for different crystals, used in roentgen spectroscopy, are found in the monograph by SIEGBAHN [88, pp. 42—48].

C. Roentgen Spectrographs with Bent Crystals

When monochromatic roentgen radiation is produced by the diffraction in *plane crystals*, the primary polychromatic roentgen beam must be restricted by narrow slits. Only a small area on the surface of the crystal takes part in the diffraction, and the resulting intensity of monochromatic roentgen radiation is very small as compared with the intensity of the primary beam. Therefore, several methods have been invented to obtain a higher yield of monochromatic roentgen radiation. DUMOND and KIRKPATRICK [26], introduced the multiple crystal roentgen ray spectrograph, but later methods, utilizing *bent crystals*, have been more advantageous. A discussion of these methods has been published by ORTNER [82].

In the soft roentgen region two procedures, utilizing bent crystals, have proved to be valuable. The first method, described by JOHANN [50] in 1931, is schematically illustrated in Fig. 13, which is a cross section through the centre of a bent crystal $C_2C_1C_3$, perpendicular to the surface of the crystal and to the diffracting atomic planes. The crystal is bent cylindrically to a radius of curvature $EC_1 = R$. The focal spot of a roentgen tube is situated at $A_1A_2A_3$ on the circumference of the *Rowland circle*, i.e. the circle with

the radius $DC_1 = \frac{R}{2}$, which is a tangent to the surface of the crystal at C_1 .

The central ray A_1C_1 in the divergent roentgen beam originating from A_1 , is incident upon the crystal at C_1 at a glancing angle φ . The monochromatic roentgen beam C_1B_1 , diffracted in the crystal according to Bragg's law, cuts the Rowland circle in B_1 . The angles A_1C_1E , $A_1C'_2E$, and $A_1C'_3E$ are circumferential angles on the same arc and are consequently identical. Therefore, if the diffracting atomic planes had cut the Rowland circle at C'_2 and had had a radius of curvature EC'_2 with the centre in E , as indicated by the dash arc in Fig. 13, the polychromatic roentgen beam $A_1C'_2$ had also been incident on the atomic planes at the glancing angle φ . A diffracted monochromatic roentgen beam C'_2B_1 had had the same wavelength as the beam C_1B_1 , and the corresponding discussion holds for a hypothetical diffracted beam C'_3B_1 . Thus a monochromatic roentgen beam, converging towards B_1 had been obtained from a polychromatic roentgen beam, originating from A_1 . However, the extensions of EC'_2 and EC'_3 cut the surface of the crystal at C_2 and C_3 , respectively. The polychromatic roentgen beam A_2C_2 , parallel with $A_1C'_2$, has the glancing angle φ , and the corresponding diffracted beam C_2B_2 has the

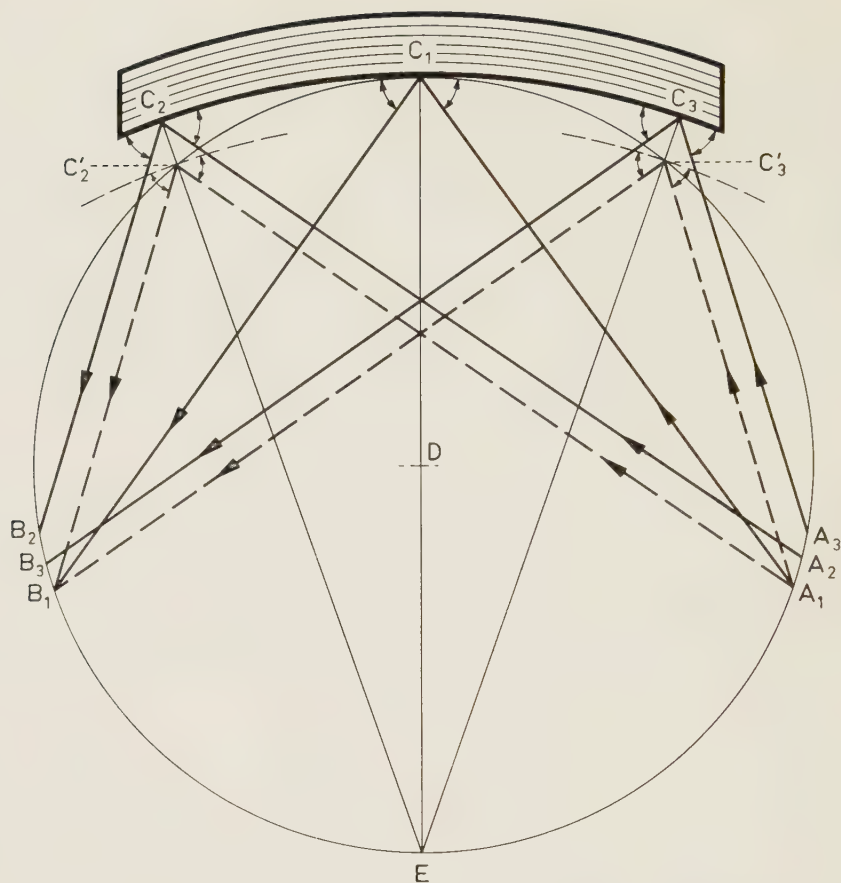


Fig. 13. The bent crystal method according to Johann. All angles marked in the figure have the value φ .

same wavelength as the beam C_1B_1 , which also holds for the diffracted roentgen beam C_3B_3 .

When utilizing the method of bent crystal according to Johann, the defective focusing results in a broadening of a roentgen emission line, generated at $A_1A_2A_3$ and diffracted by the crystal. This broadening effect $B_1B_2 = b$, measured along the focusing circle or along its tangent, tends *in the direction of shorter wavelengths*. It is given by the following expression, calculated by JOHANN [50]:

$$b = \frac{o^2}{8R} \cot \varphi \quad \dots \dots \dots (42).$$

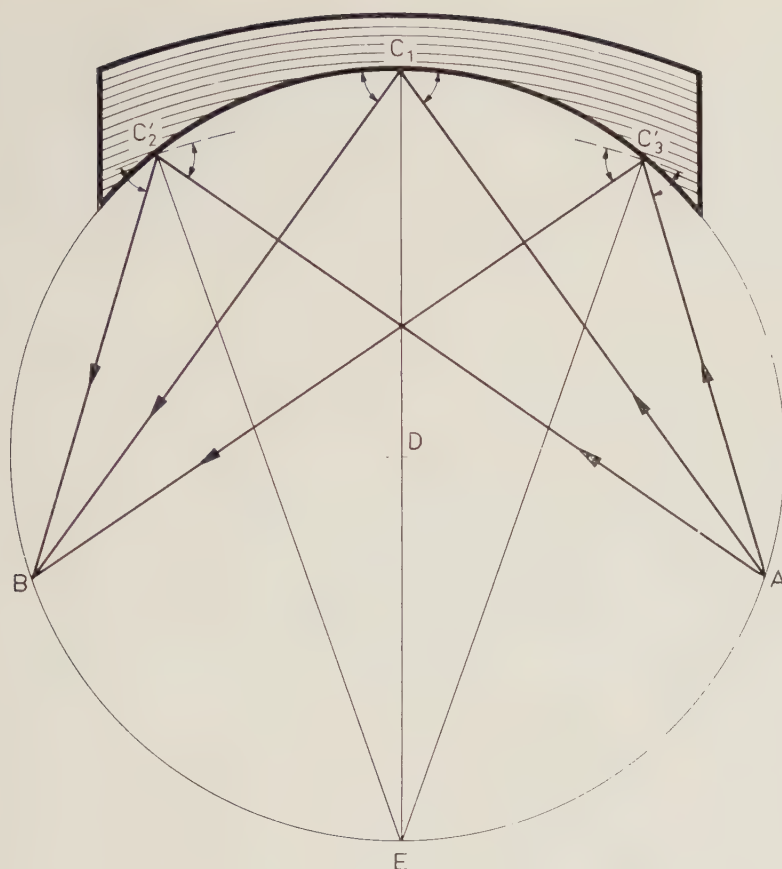


Fig. 14. The bent crystal method according to Johansson. All angles marked in the figure have the value φ .

o is the linear opening (mm) of the crystal, R the radius of curvature (mm) of the crystal, and φ the glancing angle. Other broadening effects are discussed in Part III of this work in connection with the high vacuum roentgen ray spectrograph, designed for elementary analyses on a cellular level.

The second method, utilized by JOHANSSON [51], is schematically illustrated in Fig. 14. The crystal $C_2C_1C_3$ is bent cylindrically to a radius of curvature $EC_1 = R$, and is then ground cylindrically to a radius of curvature $DC_1 = \frac{R}{2}$. Thus the surface of the crystal follows the Rowland circle along the whole opening of the crystal. This corresponds exactly to the hypothetical

case discussed in connection with the Johann method. The divergent roentgen beam from A is incident upon the atomic planes, marked in Fig. 14, with a *constant* glancing angle, and the diffracted, monochromatic roentgen rays are focused exactly in the point B. However, the technical difficulties involved in the exact grinding of a bent crystal are considerable, and few crystal materials can be used. Therefore, only roentgen ray spectrographs with bent crystals according to Johann have been extensively utilized.

D. The Diffraction of Roentgen Rays by Ruled Gratings

According to Bragg's law the wavelength of roentgen radiation, diffracted by a crystal, must be smaller than twice the utilized grating space. Mica has the largest grating space ($d_1 = 9927.58$ X.U.) [88, p. 48] among the naturally occurring crystals, used in roentgen spectroscopy. The upper wavelength limit of roentgen radiation, diffracted by the usual crystals, is then about 19000 X.U. However, some higher fatty acids and different metal compounds of these acids have larger grating spaces, but there are considerable difficulties to make crystals of these chemical compounds for purposes of roentgen spectroscopy.

As roentgen radiation, incident upon surfaces of solids, is totally reflected, provided the incident glancing angle is smaller than the critical grazing angle, roentgen rays can be diffracted by reflection from ruled gratings. The roentgen rays are scattered at the grooves, ruled in the grating, and the scattered radiation is intensified in certain directions by constructive interference.

Concave gratings with focusing properties are generally used instead of plane gratings, from which the scattered radiation is very weak. Detailed discussions of the properties of *concave gratings* have been published by MAGNUSSON [74] and TYRÉN [95]. The principle of a concave grating with a radius of curvature R is shown in Fig. 15, which is a cross section at right angles to the ruled grooves. The roentgen radiation, incident on the grating $G_2G_1G_3$, is restricted by a narrow slit S, placed on the circumference of the Rowland circle, which has the radius $\frac{R}{2}$. For the central ray SG_1 the incident glancing angle Θ (exaggerated in Fig. 15) is smaller than the critical grazing angle Θ_c , which is determined according to equation (39 a). The totally

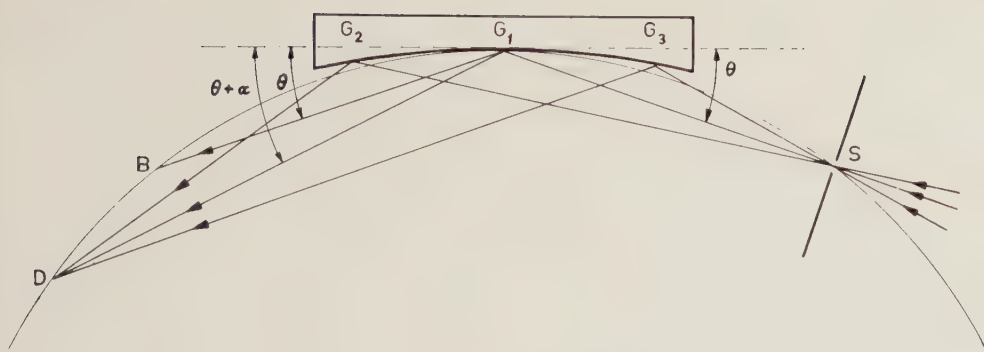


Fig. 15. The principle of the concave grating.

reflected beam intersects the Rowland circle at B, and the diffracted beam with the glancing angle $\Theta + \alpha$ and the wavelength λ cuts the focusing circle at D. Roentgen rays, diffracted from other points on the concave grating, e.g. from G_2 and G_3 , are focused at the same point D on the circumference of the Rowland circle, provided they have the same wavelength λ as the diffracted beam G_1D .

The relation between the different quantities is given by the grating equation

$$n\lambda = d [\cos \Theta - \cos (\Theta + \alpha)] \quad \dots \dots \dots (43),$$

where n is the order of diffraction, and d the distance between adjacent grooves. The other symbols have been defined in the preceding text. λ and d are expressed in Å.U. According to equation (43) the *absolute* wavelengths of roentgen emission lines can be accurately determined. The discrepancy between the *crystal scale* with the unit 1 X.U. and the *absolute scale* with the unit 1 Å.U. can be determined in this way.

The Measurement of the Intensity of Roentgen Rays

When applying roentgen absorption spectrophotometry in quantitative cytochemistry, the intensity of the roentgen radiation can be registered by means of photographic procedures, by utilizing the ionizing effect of the roentgen rays during the passage through different gases, or by the use of a scintillation counter. In the field of cytochemistry a registering method must be chosen to make possible an accurate localization of the analysed structure. In this respect the use of fine-grained photographic emulsions are superior to other methods. When measuring the intensity of a roentgen beam, however, the methods utilizing the ionizing effect of the roentgen rays and the scintillation counter are much more sensitive and accurate and should therefore be used, when the problem of localization does not preclude their application.

A. The Photographic Action of Roentgen Rays

The density D of a photographic emulsion is given by the expression

$$D = \log \frac{i_0}{i_1} \dots\dots\dots (44),$$

where i_0 and i_1 are the intensities of visible light transmitted through an unexposed area and through the exposed area, respectively, in the developed film emulsion. D is then the density of an exposed area when the density of the fog has been subtracted.

If the photographic emulsion is exposed to visible light, the *Bunsen-Roscoe reciprocity law*

$$D = f(It) \dots\dots\dots (45)$$

is not valid. In this region of the electromagnetic spectrum the relation between the density D , the intensity I of the radiation incident upon the photographic emulsion, and the time of exposure t can be expressed according to the Schwarzschild law :

$$D = f(It^n) \dots\dots\dots (46).$$

For limited ranges of the intensity I the exponent p is a constant and has a value less than unity at low intensities.

A series of investigations have verified the *Bunsen-Roscoe reciprocity law* for roentgen radiation [38, 6, 7, 4, 78] and *no intermittency effect* has been found [4]. BELL [4] confirmed the validity of the law over an intensity range of 10^4 to 1. MÜLBACH [78] found the Schwarzschild exponent to be unity for photographic emulsions exposed to soft roentgen radiation, the La_1 line of silver ($\lambda = 4146$ X.U.) and the Ma_1 line of tungsten ($\lambda = 6960$ X.U.).

For ordinary roentgen radiation with wavelengths between ~ 100 and ~ 2000 X.U. there is no initial lag in the response of photographic emulsions to different exposures. *When the density of the developed emulsion is plotted as a function of the exposure*, i.e. the product of the intensity I of the incident roentgen radiation and the time of exposure t , *the curve has an initial straight portion* passing through the origin of the system of coordinates, which has been confirmed in many investigations [33, 38, 6, 10, 7, 4]. The extension of the straight portion of the curve varies for different photographic emulsions and for different developing conditions. For a rather wide range of wavelengths, however, the shape of the density curve is independent of the quality of the roentgen radiation [38, 6, 4].

For soft roentgen radiation the shape of the density curves is more complicated with transitions to the general shape found for photographic emulsions exposed to visible radiation. Thus BROILI and KIESSIG [8] found that the density plotted against the time of exposure for the $K\alpha$ line of carbon ($\lambda \approx 44500$ X.U.) had a curved shape with a very short initial straight portion, and an initial lag of response was obtained for one emulsion. For the same photographic emulsions the corresponding curves for the $K\alpha$ line of copper ($\lambda \approx 1540$ X.U.) had a much longer region of linearity. For an emulsion exposed to the La_1 lines of Cd, Rh, Ru, and Mo (wavelengths from 3948 X.U. to 5395 X.U.) HIRSCH [44] obtained an initial straight portion, when plotting the density versus the time of exposure. Corresponding curves, obtained for roentgen radiation in the region between 11000 and 14000 X.U., were not linear [45]. MÜLBACH [78] found no sensitivity threshold in the photographic response to the La_1 line of silver ($\lambda = 4146$ X.U.) and to the Ma_1 line of tungsten ($\lambda = 6969$ X.U.). For the latter roentgen radiation the density of the photographic emulsion was a linear function of the exposure up to a density of 0.5. For a Printon emulsion, exposed to the $K\alpha$ line of

aluminium ($\lambda = 8320$ X.U.), ENGSTRÖM [29] found the density to be a linear function of the intensity only up to a density of 0.15.

It is assumed that, within a certain space of time, a minimum of energy must be absorbed in a grain in a photographic emulsion to make the grain developable. This explains the failure of the reciprocity law for low intensities of visible radiation, as several quanta must be absorbed in a grain to make it developable at such low quantum energies. For heterogeneous roentgen radiation with an effective wavelength of 450 X.U. the quantum yield at the photolysis of silver bromide was about 1000 silver atoms per quantum, and each absorbed quantum made approximately one grain developable [27, 28]. In an investigation by SILBERSTEIN and TRIVELLI [89] the grains in an emulsion were classified according to their size, and after exposure to roentgen radiation the percentages of developable grains in the different classes were determined. The distribution of the values was consistent with the one calculated theoretically with the assumption that each absorbed roentgen quantum made one grain developable. The validity of the reciprocity law and the lack of sensitivity threshold for roentgen radiation are further evidence of this assumption. In a series of investigations [83, 93, 47, 48, 9, 87] the average number of developable grains per absorbed roentgen quantum was found to be constant in a wavelength range around 1000 X.U., i.e. independent of the wavelength, but increased continuously at higher quantum energies.

GLOCKER [35, 36] has stressed that the total kinetic energy of the electrons, liberated in the photographic emulsion by the absorbed roentgen quanta, determines the photolytic process. For roentgen radiation in the wavelength range from 240 to 1540 X.U., however, only 5 or 6 per cent of this energy was utilized to produce silver atoms [40]. In an investigation of the spectral sensitivity of photographic emulsions between 200 and 2500 X.U. SEEMAN [87] found that *the absorbed roentgen energy was utilized more efficiently for soft roentgen radiation*, although the grain yield was greater for roentgen quanta with high quantum energies. For a detailed discussion of these problems, and of the photographic effect of roentgen rays in general, reference should be made to CHARLESBY [14] and KORNFELD [58].

The majority of investigations of the photographic action of roentgen rays has been carried out at wavelengths shorter than about 2500 X.U. In roentgen absorption spectrophotometry applied in cytochemistry this is the short wavelength limit of the roentgen radiation to be utilized. The few investigations

with soft roentgen radiation have shown the validity of the Bunsen-Roscoe reciprocity law, the absence of an intermittency effect, the initial linearity between the photographic density and the exposure, and the increasing photographic effect of the absorbed roentgen radiation at longer wavelengths. Furthermore, the composite mass absorption coefficient of the silver bromide emulsion is approximately proportional to the third power of the wavelength, with the exception of the discontinuities at the absorption edges of silver and bromine. Therefore, between the absorption edges, the photographic effect of roentgen radiation, *incident* upon the photographic emulsion, increases parallel with the fraction of absorbed roentgen energy towards longer wavelengths.

B. Methods Based upon the Ionizing Effect of Roentgen Rays on Gases

The ionizing effect of roentgen radiation is measured by means of a gas-filled chamber, consisting of a cylindrical cathode and an axially mounted wire which serves as anode. The roentgen quanta, absorbed in the gas volume within the counter, generate photoelectrons, which dissipate their kinetic energy by ionizing other gas molecules. If the voltage across the chamber exceeds a certain value, all the ions and electrons are collected at the electrodes. The ionization current then reaches a saturation value, and the chamber acts as an *ionization chamber* with an amplification factor of unity. The ionization current is a measure of the absorbed roentgen intensity, and not of the incident roentgen intensity. Therefore, different ionization currents are obtained for incident roentgen beams with the same intensities but with different wavelengths, owing to the difference between the mass absorption coefficients.

With increasing voltage across the chamber, the electrons, generated in the primary ionizing process, gradually acquire higher kinetic energies, especially in the vicinity of the central wire, where the electric field is strongest. When the kinetic energy of the primary electrons exceeds the value necessary to ionize new gas molecules, the chamber begins to act as a *proportional counter*. Owing to secondary ionization of gas molecules the amplification factor increases with increasing voltage and can amount to 10^4 or 10^5 . For a constant voltage the output pulse is linearly proportional to the energy of the absorbed roentgen quantum for a wide range of quantum energies, if nitrogen or argon are utilized in the chamber [21].

When the voltage across the tube amounts to about 1000 or 1500 volts, the primary electrons acquire sufficient energy to excite radiation quanta with frequencies in the far ultraviolet and in the extremely soft roentgen region. These quanta release new photoelectrons, and the discharge spreads along the whole wire. The space charge of slowly moving positive ions counteracts the strong electric field around the central wire and terminates the discharge. The chamber then acts as a *Geiger-Mueller counter* with an amplification factor in the range between 10^8 and 10^{10} .

Self-quenching counters are generally filled with a mixture of an inert gas and a polyatomic vapour, e.g. a mixture of argon and ethyl alcohol. During the discharge some atoms in the inert gas are brought to metastable states and can therefore initiate a new discharge. These atoms are de-excited by organic molecules, which are decomposed without any formation of new secondary electrons [57]. Self-quenching counters filled with a gas mixture composed of neon, argon, and a halogen, operate at about 400 volts [72], which is a value about three to four times lower than the working voltage of a chamber, filled with argon and an organic vapour. Non-self-quenching counters must be connected with an electronic quenching circuit.

In a voltage range comprising from one to several hundred volts, called the plateau of the chamber, the response of the Geiger-Mueller counter shows a very small increase with increasing voltage across the chamber, when the number of incident roentgen quanta per unit of time is constant. During the deionization time the positive space charge around the central wire makes the Geiger-Mueller counter insensitive to absorbed roentgen quanta, which limits the response of the chamber at high pulse frequencies. Therefore, the registered number of quanta is too low at high counting rates. With linearity of response the proportional counter can operate at pulse frequencies 100 to 1000 times higher than those possible to obtain with the Geiger counter, as the positive space charge only makes a small part of the proportional counter insensitive.

For reviews of the basic principles of these different types of counters reference should be made to FRIEDMAN [32], WARMOLTZ [105], and CURRAN [20].

C. A Method Based upon the Fluorescent Effect of Roentgen Rays

The fluorescent effect of roentgen quanta is utilized in the *scintillation counter*, which consists of a photomultiplier tube, combined with a fluorescent material, such as anthracene, stilbene, or sodium iodide activated with thallium. In the fluorescent crystal incident roentgen quanta produce scintillations which are directed towards the photoelectric surface in the photomultiplier tube. The visible radiation quanta release photoelectrons which are multiplied in a series of dynodes. The potential difference between adjacent dynodes is up to 100 volts. The anode current, in the form of short pulses, is transferred to a registering circuit, sometimes via an external amplifier. As some of the organic fluorescent crystals have pulse decay times of about 10^{-8} sec., extremely high counting rates can be utilized. The frequency of thermal electrons, emitted from the photoelectric surface in the photomultiplier tube, determines the minimum anode current to be registered. The maximum anode current is restricted by fatigue of the photomultiplier tube. Detailed discussions of the scintillation counter have been published by MARSHALL *et. al.* [76, 75].

PART II

Total Dry Weight Determinations of Cytologic Structures
by Absorption Spectrophotometry Utilizing Soft
Continuous Roentgen Radiation

CHAPTER 5

Earlier Biologic Applications of Microradiography with Special Reference to Total Dry Weight Determinations

A. Microradiography with Secondary Magnification

The utilization of roentgen radiation to obtain pictures of small biologic objects was introduced by GOBY [149, 150], who in 1913 published roentgen absorption images of *Foraminifera* and introduced the term "microradiography". However, pictures of minute structures could not be obtained, as only relatively coarse-grained photographic emulsions were available. Furthermore, the utilized roentgen radiation was too hard to give adequate contrast, i.e. the absorption of the radiation in small biologic objects was too low.

Further improvements of the microradiographic procedures were possible, when fine-grained photographic emulsions were introduced, and when the specimen was placed in the evacuated part of the roentgen tube. Thus DAUVILLIER [135] obtained a roentgen absorption image of a microtome section of elder-pith, utilizing a fine-grained Lippmann emulsion and a cassette, filled with hydrogen, or evacuated to reduce the absorption of the soft roentgen radiation. The biologic specimen was in close contact with the photographic emulsion, and the *primary microradiogram* was accordingly registered in the scale 1:1 and secondarily enlarged by photo-micrography.

LAMARQUE [159] designed a special, continuously evacuated roentgen tube with a thin lithium window to obtain soft roentgen radiation. The specimen holder with a plate changer was sealed to the tube, and could be evacuated to a high vacuum simultaneously with the roentgen tube. In collaboration with TURCHINI [161, 181, 160] the technique was applied to study the roentgen absorption in numerous normal and pathologic mammalian tissues, and the term "historadiography" was introduced. However, no quantitative interpretations of the roentgen absorption images were made.

With an improved technique historadiographic studies have been performed on various tissues, i.a. thyroid [147] and skin [171]. Extensive historadiographic investigations have been made of the distribution of mineral salts in

bone tissue, i. a. by Sissons [178], and especially by ENGSTRÖM *et al.* [109, 138].

A special field in microradiography is its application to soft tissues impregnated with substances containing heavy metals. Microradiography has been applied to this type of material, i.a. by CASTEL, LAMARQUE and TURCHINI [123], by BARCLAY [111] to demonstrate iron in cells, and by MITCHELL [168, 169] to tissues, impregnated with silver.

Roentgen absorption techniques in the micro-scale have also been utilized to study the vascularization in various tissues by means of introducing roentgen contrast media in the vascular system [115, 180, 110, 112, 114, 113]. For a review of this field of microradiography, called microangiography, reference should be made to BELLMAN [113].

A special technique of microradiography has been used by EHRENBURG and SPEAR [137]. A roentgen tube with a fine focus is operated at 40 to 50 kV, and the short wavelength part of the roentgen spectrum is cut out by total reflection at an optical flat, as the critical grazing angle is proportional to the wavelength of the incident roentgen radiation (see equation (39 a)). The resolution was estimated at 0.5μ in the secondarily magnified microradiogram of a diatom fragment, registered at a wavelength of about $7.5 \text{ \AA.U.}$

For further information regarding microradiographic techniques and applications on biologic material the reader is referred to reviews by CLARK [124; 15, pp. 238—262] and ENGSTRÖM [139, 140].

B. Microradiography with Primary Magnification

The microradiographic procedures, discussed in the previous section, were based upon roentgen absorption images, registered in the scale 1:1 on fine-grained photographic emulsions and magnified *secondarily* by photo-micrography. During the last decades different procedures have been introduced to obtain *primarily magnified* images, utilizing roentgen radiation [182]. Only the shadow roentgen ray microscope and the real roentgen ray microscope according to KIRKPATRICK and BAEZ [157] will be briefly discussed in this section.

If the sample is placed in front of a point source of roentgen radiation, a *magnified shadow image* of the specimen can be registered on a photo-

graphic emulsion at a distance from the sample. The important factor, determining the resolution of the structures in the object, is the dimension of the focal spot. SIEVERT [177] suggested that a very small aperture in a screen, situated between the roentgen tube and the specimen, could serve as a point source of roentgen rays, but the intensity of the roentgen radiation is then very small.

Utilizing magnetic electron lenses to focus the electrons in the roentgen tube to a very small area in the target, COSSLETT *et al.* [130, 131, 132, 133, 134] have developed a *roentgen ray shadow microscope* with a point source of roentgen rays less than $1\ \mu$ in diameter. The anode is a part of the wall of the roentgen tube and consists of a tungsten foil ($1\ \mu$ thick), through which the divergent micro-beam traverses. Shadow microradiograms, with a primary magnification of up to 150 times, have been published of whole frozen-dried insects and of metal grids with extremely small meshes. The utilized roentgen radiation had a wavelength of about $2\ \text{\AA.U.}$

The principles of a primarily magnifying optical system for roentgen radiation were given by KIRKPATRICK and BAEZ [157], who also constructed the *first roentgen ray microscope*. The basic optical unit consisted of two concave, cylindrically ground mirrors, placed in series and crossed at right angles to each other. A divergent roentgen beam from a point, incident in turn on the two mirrors at a glancing angle smaller than the critical grazing angle, is totally reflected and converges to one point. Improved results were obtained with a compound roentgen ray microscope, consisting of two two-mirror systems with spherical surfaces [156, 174]. The calculated resolution was about $70\ \text{\AA.U.}$ [157], but from later calculations of the optimum conditions in a practical instrument a value of about $2000\ \text{\AA.U.}$ was given [176]. Recently a scanning roentgen ray microscope has been proposed [175].

So far no studies of thin sections of biologic objects have been published, applying the techniques of the roentgen ray shadow microscope or the compound roentgen ray microscope.

C. Quantitative Microradiographic Determination of the Total Dry Weight of Small Biologic Objects

In 1949—50 ENGSTRÖM and LINDSTRÖM [142, 30] published a method to determine the dry weight of small biologic objects in thin microtome sections of tissue or in smear preparations. Continuous, soft, filtered roentgen radia-

tion, transmitted through the specimen and through a reference system, consisting of a step wedge of nitrocellulose foils, is registered on a fine-grained photographic emulsion in close contact with the sample. The weight per unit area of biologic specimens can be calculated from a comparison between the densities of the absorption images of cellular structures and of the reference system in the developed microradiogram.

This quantitative microradiographic procedure, schematically represented in Fig. 16, is based on the fact that carbon, nitrogen, and oxygen in tissue proteins are the main portion of roentgen absorbing material in different biologic structures. The absorption of the continuous roentgen radiation was calculated for a mixture of carbon, nitrogen, and oxygen with the same proportions as in proteins [30], as other elements are present in relatively low concentrations in most biologic objects. The values of the mass absorption coefficients of these other elements differ from those of the C-N-O mixture, which introduces a systematic error. Utilizing monochromatic roentgen radiation at seven different wavelengths the highest allowable percentages of these elements were calculated, when the systematic error was ± 5 per cent [30]. Approximate, integrated values of the allowable concentrations of the different elements, occurring in biologic structures, were determined when utilizing a continuous roentgen spectrum, generated at 3 kV and filtered through 9μ aluminium. The total systematic error for each element was fixed at ± 5 per cent, but the utilized method of averaging gave only roughly approximate results. With the exception of hydrogen, for which element a correction factor was introduced, the highest allowable percentages of the elements exceeded the concentrations generally found in soft biologic specimens. However, the important question regarding the systematic errors, introduced by common organic compounds in biologic material, was not considered.

As the reference system is exposed simultaneously with the biologic specimen, the roentgen intensities, incident on the two objects, are equal. By photometry the transmitted roentgen intensities, registered in the developed microradiogram, are obtained as a function of the weight per unit area of the different steps in the wedge. The photo-currents corresponding to the steps in the wedge are plotted as a function of the weight per unit area of the different steps (see Fig. 16). By means of this calibration curve the photometer deflections for biologic structures are transformed to the corresponding weights per unit area of the reference system ($u_1 w$ and $u_2 w$,

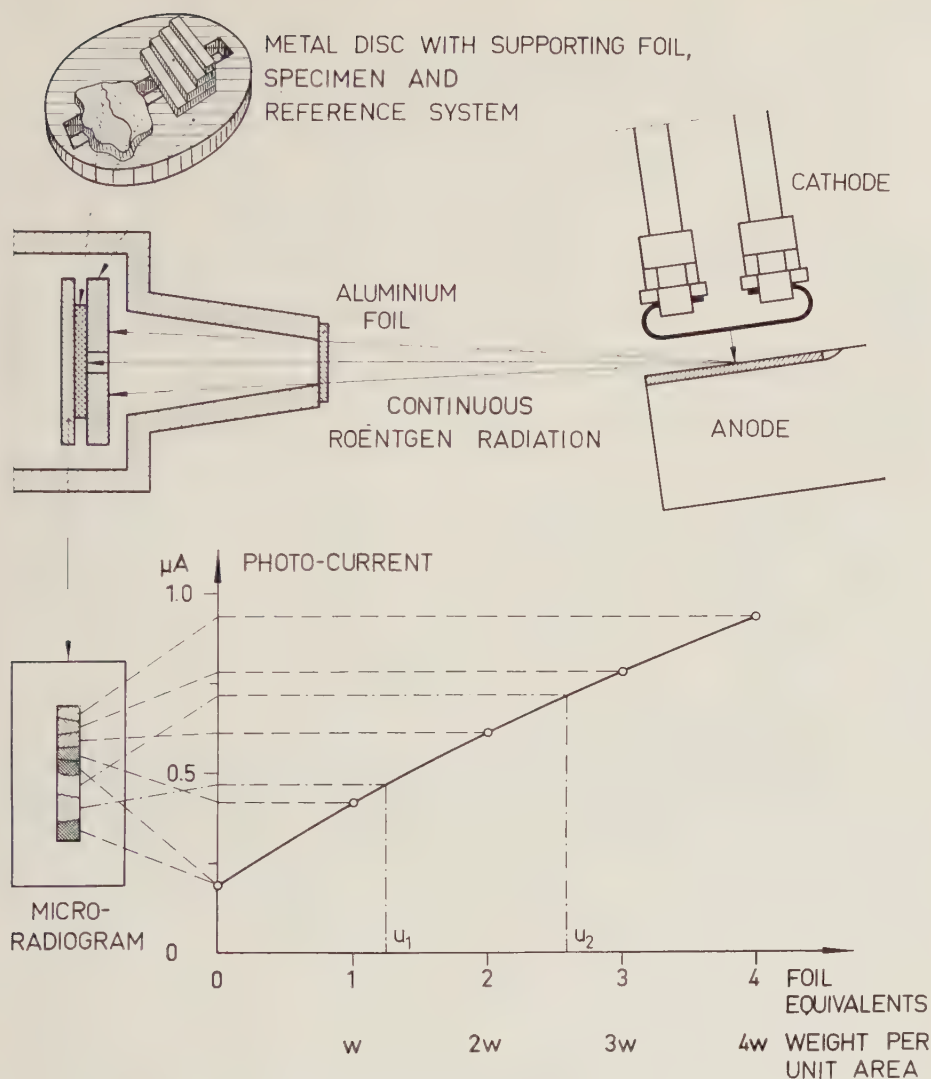


Fig. 16. Schematic representation of the quantitative microradiographic procedure to determine the total dry weight of small biologic objects.

respectively, in Fig. 16). The weight per unit area of a biologic object was obtained according to the following expression (equation 10 a in [30]) :

$$\frac{m_a}{k_{H_p}} = \frac{k_{H_{ref}}}{k_{H_p}} \cdot m_{ref} \cdot \frac{\left(\frac{\mu}{\rho}\right)_{CNO_{ref}}}{\left(\frac{\mu}{\rho}\right)_{CNO_p}} \quad (47)$$

$\frac{m_a}{k_{H_p}}$ = the weight per unit area of the biologic structure.

m_{ref} = the weight per unit area of the reference system with the same absorption as the biologic structure.

k_{H_p} = the correction factor for hydrogen in proteins.

$k_{H_{\text{ref}}}$ = the correction factor for hydrogen in the reference system.

$\left(\frac{\mu}{\rho}\right)_{\text{CNO}_p}$ = the mass absorption coefficient of a C-N-O mixture with the same proportions as in proteins.

$\left(\frac{\mu}{\rho}\right)_{\text{CNO}_{\text{ref}}}$ = the mass absorption coefficient of the C-N-O mixture in the reference system at the same wavelength.

Utilizing the original technique [30], the distribution of the total dry weight in different biologic objects has been investigated, i.a. the dry weight of different gastric mucosa cells [141], the distribution of the dry weight and of lipids in the myelin sheath of nerve fibres [144], the dry weight of ventral horn cells of the cat [173], and the dry weight of giant chromosomes from *Chironomus* [145]. The errors of the original method have been discussed by LINDSTRÖM [163, 164].

With a modified technique BRATTGÅRD, HYDÉN *et al.* [121, 117, 152, 151, 136, 116] have adopted this microradiographic procedure to extensive cytochemical studies of the nervous system. The errors of this modified technique have been discussed in several papers [118, 119, 120]. These investigations have been reviewed by BRATTGÅRD and HYDÉN [122]. Cytochemical studies of the human ovarian oocyte have been made with the same technique [153].

CLEMMONS *et al.* [126, 125, 128, 127] have also constructed a roëntgen equipment and have modified the original technique to study, especially, the distribution of the dry weight in cartilage during the early stages of calcification. Lately COMBÉE and ENGSTRÖM [129] have introduced a new device for microradiography and a technique where a reference system is not utilized.

CHAPTER 6

The Theoretical Basis

In the following Chapters 6—9 the quantitative microradiographic method to determine the total dry weight of small biologic objects will be described. The generalized theoretical basis is presented, followed by a description of the experimental apparatus and technique, as developed by the author during the course of several years [165]. The accuracy and resolution of the method are discussed in detail, and in the last chapter of Part II certain applications of the method are presented.

In the original presentation of the method [30] the voltage across the roentgen tube was 3 kV, corresponding to a short wavelength limit of 4.12 kX.U. according to equation (4a). An aluminium foil with a thickness of $9\ \mu$ selectively absorbed roentgen radiation with wavelengths shorter than the K absorption limit of aluminium at 7.94 kX.U. Therefore, roentgen radiation in the wavelength range between 8 and 12 kX.U. comprised about 80 per cent of the intensity of the continuous, filtered roentgen spectrum.

The accuracy and the resolution of the method will be improved, and the range of applications will increase, when the continuous roentgen spectrum is generated at a voltage lower than 3 kV, which has been pointed out previously [163]. When utilizing extremely soft radiation a high contrast can also be obtained in roentgen absorption images of extremely thin sections of biologic specimens. Because of the discontinuous decrease of the mass absorption coefficient of oxygen at the K absorption limit of this element at 23.35 Å.U. [74], the absorption of roentgen radiation in biologic specimens decreases at that wavelength. Therefore, 23 kX.U. can be considered as the suitable long wavelength limit of the radiation in the continuous roentgen spectrum to be utilized.

A. Mass Absorption Coefficients of the Elements with Low Atomic Numbers and of Some Common Organic Compounds in Biologic Material

The experimentally determined values of the mass absorption coefficients are rather divergent, especially for soft roentgen radiation, as discussed in

Table IV. Mass absorption coefficients

Element	2.5 kX.U.	3.0 kX.U.	3.5 kX.U.	4.0 kX.U.	5.0 kX.U.	6.0 kX.U.	7.0 kX.U.	8.0 kX.U.	9.0 kX.U.
1 H	0.55	0.74	0.96	1.22	1.98	3.1	4.7	6.9	9.6
2 He	1.00	1.58	2.4	3.6	6.6	11.2	18.1	27	39
3 Li	2.5	4.2	6.6	9.7	18.6	32	51	75	107
4 Be	5.9	10.0	15.5	23	44	76	120	178	260
5 B	9.3	15.9	25	37	71	125	190	290	400
6 C	19.0	34	54	80	153	250	400	600	830
7 N	30	54	86	130	250	420	660	960	1350
8 O	48	84	133	200	380	630	980	1400	1940
9 F	67	116	182	270	500	840	1280	1850	2500
10 Ne	96	165	260	370	690	1130	1720	2500	3400
11 Na	122	200	320	470	850	1390	2100	3000	4000
12 Mg	160	270	410	600	1100	1780	2600	3600	4700
13 Al	195	330	500	720	1300	2100	3100	4200	5500
14 Si	240	400	600	880	1590	2500	3600	4800	6200
15 P	280	480	730	1040	1800	2600	3900	5500	7800
16 S	360	580	880	1250	2200	3300	5000	7200	10000
17 Cl	400	660	980	1400	2600	4100	6200	8900	12200
18 A	460	760	1160	1650	3000	5000	7600	11000	15000
19 K	540	850	1400	2000	3700	6000	9100	13100	17600
20 Ca	620	960	1700	2400	4400	7000	10600	15000	20000
22 Ti	105	160	240	330	580	920	1360	1900	2500
24 Cr	135	210	300	420	730	1130	1660	2300	3000
26 Fe	150	240	360	520	880	1340	1930	2600	3400
28 Ni	180	290	430	590	1000	1500	2100	2900	3700
29 Cu	200	330	490	690	1150	1720	2400	3200	4200

Chapter 2: E. The mass absorption coefficients of elements with low atomic numbers have been compiled in Table IV, which comprises the whole suitable wavelength region to be utilized for determinations of the total dry weight of small biologic objects. All the common elements in biologic material are included. The main sources for the compilation have been Allen's tables [16, pp. 799—806; 46] and the values calculated by VICTOREEN [99, 100]. The values of each element were plotted in a double logarithmic coordinate system, where an approximately linear relationship holds between the mass absorption coefficient and the wavelength of the roentgen radiation (see Fig. 4). The

of elements with low atomic numbers

10.0 kX.U.	11.0 kX.U.	12.0 kX.U.	13.0 kX.U.	14.0 kX.U.	15.0 kX.U.	16.0 kX.U.	18.0 kX.U.	20.0 kX.U.	22.0 kX.U.
13.1	17.4	22	28	35	43	52	74	102	135
53	70	91	115	144	177	210	300	400	540
145	192	250	310	390	480	580	820	1120	1470
350	460	590	750	930	1130	1350	1900	2600	3400
560	720	950	1190	1500	1820	2200	3800	4100	5400
1120	1460	1850	2300	2800	3400	4000	5500	7300	9400
1820	2400	3000	3800	4500	5500	6400	8800	11300	14400
2500	3300	4200	5100	6200	7400	8500	11500	14900	18500
3600	4200	5200	6400	7600	8700	10000	12500	1250	1700
4400	5500	6800	8000	9400	720	860	1280	1750	2400
5100	6300	560	690	810	1030	1200	1750	2400	3200
440	580	760	940	1130	1400	1650	2300	3200	4200
590	770	1000	1240	1530	1860	2200	3100	4200	5500
780	1030	1310	1630	2000	2400	2900	4000	5300	7000
1020	1350	1680	2100	2600	3100	3700	5100	6700	8800
1300	1690	2100	2700	3300	3900	4600	6400	8400	10800
1620	2100	2600	3300	4000	4800	5600	7700	10000	13000
1950	2500	3200	3900	4800	5700	6800	9300	12300	15800
2300	3000	3700	4700	5500	6700	7800	10800	14000	16500
2600	3400	4200	5200	6100	7500	8800	12000	15500	20000
3300	4200	5100	6400	7300	9200	10700	14500	19000	24000
3800	4800	5800	7100	8200	10000	12000	16200	—	4000
4300	5300	6400	7600	8800	—	—	3000	3700	4600
4700	5800	7000	—	—	2300	2700	3500	4300	5300
5100	6200	—	—	2300	2700	3100	3900	4900	6000

values in Table IV were read off from the curves, obtained for the different elements. Interpolations between different elements were performed at certain wavelengths in those cases where values could not be found in the physical literature. No attempt was made to evaluate mass absorption coefficients at wavelengths between the L_I and L_{III} absorption limits of the absorbing element.

When the elementary composition of a chemical compound is known, the composite mass absorption coefficient for the compound can be calculated according to equation (22). *Different kinds of proteins have a remarkably*

Table V. The elementary composition of some common organic compounds

Compound	% C	% H	% N	% O	% S	% P	Reference
Animal proteins	<u>52.5</u>	<u>7.0</u>	<u>16.5</u>	<u>22.5</u>	<u>1.5</u>		[108]
Fibrin	52.68	6.83	16.91	22.48	1.10		[148]
Elastin	52.4	7.2	17.1	~23.1	0.2		[179]
Collagen	50.2	6.7	18.2	24.3	~0.6		[148]
Amyloid	<u>49.6</u>	<u>6.8</u>	<u>14.0</u>	<u>~26.8</u>	<u>2.8</u>		[108]
Thymus histone	52.37	7.70	18.35	~20.96	0.62		[108]
Plant proteins	<u>52.0</u>	<u>6.9</u>	<u>17.6</u>	<u>22.5</u>	<u>0.9</u>		[108]
Desoxyribonucleic acid	37.18	4.14	15.14	~34.60		8.94	[108]
Ribonucleic acid	<u>35.0</u>	<u>4.1</u>	<u>16.5</u>	<u>~34.7</u>		<u>9.7</u>	[108]
Glycogen	44.44	7.77		47.79			[108]
Tripalmitin	75.87	12.24		11.89			[155]
Tristearin	76.79	12.44		10.77			[155]
Triolein	77.32	11.84		10.84			[155]
Trilinolein	77.85	11.23		10.92			[155]
Cerebrosides	66.5	11.0	2.5	~18.3	0.7	1.0	[108]

constant elementary composition despite the great differences in the amino acid composition. For animal proteins the approximate ranges are 50.6—54.5 per cent carbon, 6.5—7.3 per cent hydrogen, 15.0—17.6 per cent nitrogen, 21.5—23.5 per cent oxygen, 0.3—2.2 per cent sulphur, and 0.42—0.85 per cent phosphorus [108]. The approximate elementary compositions of some common organic compounds in biologic material have been compiled in Table V. The underlined values are mean values, and the sign ~ means that the value is the difference between 100 per cent and the sum of the percentages of the other elements in that particular compound. For animal proteins the percentages of sulphur and phosphorus have been added, as the mass absorption coefficients of these elements are of the same magnitude, and as the contribution to the composite mass absorption coefficient of protein is small. The mean values for plant proteins have been calculated from 42 elementary analyses of different proteins [108], and the individual percentages of the different elements are closely spaced round the mean values.

Analyses of lipids are generally performed with regard to the percentages of different fatty acids. In animal lipids palmitic acid, stearic acid, oleic acid,

Table VI. The ratio between the mass absorption coefficients of hydrogen, carbon, nitrogen, oxygen, and sulphur at certain wavelengths

λ kX.U.	$\left(\frac{\mu}{\rho}\right)_{\text{H}}$	$;$	$\left(\frac{\mu}{\rho}\right)_{\text{C}}$	$;$	$\left(\frac{\mu}{\rho}\right)_{\text{N}}$	$;$	$\left(\frac{\mu}{\rho}\right)_{\text{O}}$	$;$	$\left(\frac{\mu}{\rho}\right)_{\text{S}}$
2.5	0.0183	:	0.63	:	1.00	:	1.60	:	12.00
3.0	0.0137	:	0.63	:	1.00	:	1.56	:	10.74
3.5	0.0112	:	0.63	:	1.00	:	1.55	:	10.23
4.0	0.0094	:	0.62	:	1.00	:	1.54	:	9.62
5.0	0.0079	:	0.61	:	1.00	:	1.52	:	8.80
6.0	0.0074	:	0.60	:	1.00	:	1.50	:	0.79
7.0	0.0071	:	0.61	:	1.00	:	1.48	:	0.76
8.0	0.0072	:	0.63	:	1.00	:	1.46	:	0.75
9.0	0.0071	:	0.61	:	1.00	:	1.44	:	0.74
10.0	0.0072	:	0.62	:	1.00	:	1.37	:	0.71
11.0	0.0073	:	0.61	:	1.00	:	1.38	:	0.70
12.0	0.0073	:	0.62	:	1.00	:	1.40	:	0.70
13.0	0.0074	:	0.61	:	1.00	:	1.34	:	0.71
14.0	0.0078	:	0.62	:	1.00	:	1.38	:	0.73
15.0	0.0078	:	0.62	:	1.00	:	1.35	:	0.71
16.0	0.0081	:	0.63	:	1.00	:	1.33	:	0.72
18.0	0.0084	:	0.63	:	1.00	:	1.31	:	0.73
20.0	0.0090	:	0.65	:	1.00	:	1.32	:	0.74
22.0	0.0094	:	0.65	:	1.00	:	1.28	:	0.75

and linoleic acid constitute the main portion of the fatty acids, while other acids are present only in smaller amounts [155]. The fatty acids are combined with glycerol in the form of esters, either triglycerides or mixed glycerides. As can be seen from Table V, the elementary compositions of the different triglycerides are very similar, and for mixed glycerides the differences in composition are still smaller.

The ratios between the mass absorption coefficients of the elements, constituting the main portion of organic material, are given in Table VI, with the mass absorption coefficients of nitrogen at different wavelengths as unit. The ratios between the mass absorption coefficients of hydrogen, carbon, nitrogen, and oxygen are approximately independent of the wavelength,

Table VII. Mass absorption coefficients

Compound	2.5 kX.U.	3.0 kX.U.	3.5 kX.U.	4.0 kX.U.	5.0 kX.U.	6.0 kX.U.	7.0 kX.U.	8.0 kX.U.
Animal proteins	31.2	54.4	85.7	127.3	240	347	547	800
Fibrin	29.9	52.4	82.6	122.9	233	348	548	802
Elastin	26.9	47.7	75.6	112.9	215	349	550	804
Collagen	28.9	50.8	80.4	120.0	228	357	562	821
Amyloid	36.6	63.2	99.2	146.6	274	361	568	828
Thymus histone	27.8	49.0	77.5	115.5	219	342	539	789
Plant proteins	29.2	51.4	81.1	120.8	229	349	549	803
Desoxyribonucleic acid	53.3	92.8	144.4	212	387	398	623	902
Ribonucleic acid	55.4	96.5	150.1	220	401	401	627	908
Glycogen	31.4	55.3	87.6	131.2	250	412	646	936
Tripalmitin	20.2	35.9	56.9	84.6	161.5	265	421	623
Tristearin	19.8	35.2	55.9	83.1	158.7	260	413	612
Triolein	20.0	35.5	56.3	83.7	159.7	262	416	616
Trilinolein	20.1	35.7	56.7	84.3	160.8	264	419	621
Cerebrosides	27.5	48.3	76.0	112.3	211	297	470	690

which has been utilized to simplify the calculations of the composite mass absorption coefficients of organic compounds [30]. If the individual mass absorption coefficients of the elements at different wavelengths are used, however, more accurate values of the composite mass absorption coefficients are obtained. The calculations of the composite mass absorption coefficients of the selected organic compounds, presented in Table VII, are based upon the values in Tables IV and V.

B. The Systematic Errors Utilizing Monochromatic Roentgen Radiation

The original approximate calculations [30] were based on the mass absorption coefficients of a C-N-O mixture with the same proportions as in an average protein. In the following investigation of the systematic errors the calculated mass absorption coefficients of an average animal protein have been selected as the basis.

When *monochromatic* roentgen radiation with the wavelength λ_i is incident upon a mixture of an average animal protein and n elements or chemical compounds x_i , the intensity of the roentgen radiation, transmitted through

of some common organic compounds

9.0 kX.U.	10.0 kX.U.	11.0 kX.U.	12.0 kX.U.	13.0 kX.U.	14.0 kX.U.	15.0 kX.U.	16.0 kX.U.	18.0 kX.U.	20.0 kX.U.	22.0 kX.U.
1111	1471	1932	2440	3020	3660	4420	5140	7030	9180	11640
1113	1475	1937	2450	3030	3670	4430	5150	7050	9210	11670
1117	1479	1942	2460	3040	3680	4440	5170	7060	9210	11690
1140	1510	1983	2510	3100	3750	4530	5270	7200	9400	11910
1149	1518	1993	2520	3120	3780	4550	5290	7230	9440	11950
1096	1454	1908	2410	2990	3610	4370	5080	6950	9080	11520
1115	1478	1941	2460	3040	3680	4440	5160	7060	9220	11690
1254	1649	2170	2750	3380	4100	4940	5730	7820	10180	12870
1262	1659	2180	2760	3410	4130	4970	5770	7870	10240	12940
1297	1693	2230	2830	3460	4210	5050	5840	7950	10370	13030
862	1149	1502	1906	2350	2870	3460	4050	5550	7320	9350
847	1131	1479	1876	2320	2820	3410	3990	5470	7220	9230
853	1139	1489	1888	2330	2840	3440	4020	5510	7270	9290
859	1146	1499	1901	2350	2860	3460	4050	5550	7320	9350
957	1269	1662	2110	2600	3160	3820	4450	6090	8000	10170

the mixture, is calculated according to equation (23). The following expression is obtained:

$$I_{1j} = I_{0j} e^{-\left(\frac{\mu}{\rho}\right)_{\text{prot}_j} m_{\text{prot}}} - \sum_{i=1}^n \left(\frac{\mu}{\rho}\right)_{x_{ij}} m_{x_i} \dots \dots \dots (48).$$

I_{0j} = the intensity of the incident roentgen radiation with the wavelength λ_j .

I_{1j} = the intensity of the corresponding transmitted roentgen radiation.

$\left(\frac{\mu}{\rho}\right)_{\text{prot}_j}$ = the mass absorption coefficient of the average animal protein at the wavelength λ_j .

$\left(\frac{\mu}{\rho}\right)_{x_{ij}}$ = the corresponding mass absorption coefficient of the element or compound x_i .

m_{prot} = the weight per unit area of the average protein.

m_{x_i} = the weight per unit area of the element or compound x_i .

If the mixture of the average protein and the elements or compounds x_i

is assumed to have the same mass absorption coefficient as an average animal protein, the following equation is valid :

$$I_{1j} = I_{0j} e^{-\left(\frac{\mu}{\rho}\right)_{\text{prot}_j} m_{\alpha j}} \dots \dots \dots (49).$$

where $m_{\alpha j}$ is the approximate weight per unit area of the mixture, when utilizing radiation with the wavelength λ_j . It has a value that equalizes the exponents in equations (48) and (49). From this assumption a systematic error, ϵ_j , is introduced, as $m_{\alpha j}$ generally differs from the correct weight per unit area. Thus the following expression holds :

$$m_{\alpha j} = (1 + \epsilon_j) \left(m_{\text{prot}} + \sum_{i=1}^n m_{x_i} \right) = (1 + \epsilon_j) m_x \dots \dots \dots (50),$$

where m_x is the correct weight per unit area.

In equations (48) and (49) the exponents are equal :

$$\left(\frac{\mu}{\rho}\right)_{\text{prot}_j} m_{\text{prot}} + \sum_{i=1}^n \left(\frac{\mu}{\rho}\right)_{x_{ij}} m_{x_i} = \left(\frac{\mu}{\rho}\right)_{\text{prot}_j} m_{\alpha j} \dots \dots \dots (51).$$

When $m_{\alpha j}$ is eliminated in equations (50) and (51), the following is obtained :

$$\left(\frac{\mu}{\rho}\right)_{\text{prot}_j} m_{\text{prot}} + \sum_{i=1}^n \left(\frac{\mu}{\rho}\right)_{x_{ij}} m_{x_i} = \left(\frac{\mu}{\rho}\right)_{\text{prot}_j} (1 + \epsilon_j) \left(m_{\text{prot}} + \sum_{i=1}^n m_{x_i} \right) \dots \dots (52).$$

After transformations the expression is

$$\sum_{i=1}^n \left(\frac{\mu}{\rho}\right)_{x_{ij}} m_{x_i} - \sum_{i=1}^n \left(\frac{\mu}{\rho}\right)_{\text{prot}_j} m_{x_i} = \epsilon_j \left(\frac{\mu}{\rho}\right)_{\text{prot}_j} \left(m_{\text{prot}} + \sum_{i=1}^n m_{x_i} \right) \dots \dots (52 \text{ a}).$$

Introducing m_x in equation (52 a) and solving for ϵ_j , gives

$$\epsilon_j = \frac{\sum_{i=1}^n m_{x_i} \left[\left(\frac{\mu}{\rho}\right)_{x_{ij}} - \left(\frac{\mu}{\rho}\right)_{\text{prot}_j} \right]}{m_x \left(\frac{\mu}{\rho}\right)_{\text{prot}_j}} \dots \dots \dots (53 \text{ a}).$$

which expresses the resulting systematic error for a mixture of an average animal protein and n elements or chemical compounds x_i , exposed to monochromatic roentgen radiation with the wavelength λ_j . The mixture is assumed to have the same mass absorption coefficient as the protein. This expression is composed of the sum of n partial systematic errors ε_{ij} , given by

$$\varepsilon_{ij} = \frac{m_{x_i}}{m_x} \cdot \frac{\left(\frac{\mu}{\rho}\right)_{x_{ij}} - \left(\frac{\mu}{\rho}\right)_{\text{prot}_j}}{\left(\frac{\mu}{\rho}\right)_{\text{prot}_j}} \dots\dots\dots (54).$$

Thus the partial systematic error ε_{ij} is proportional to the relative amount of the element or compound x_i , m_{x_i}/m_x , and to the relative difference between the mass absorption coefficients of x_i and of the average protein,

$$\left[\left(\frac{\mu}{\rho}\right)_{x_{ij}} - \left(\frac{\mu}{\rho}\right)_{\text{prot}_j} \right] / \left(\frac{\mu}{\rho}\right)_{\text{prot}_j}.$$

As $m_{x_i}/m_x \leq 1$, the partial systematic error is restricted by the expression

$$\varepsilon_{ij} \leq \left| \frac{\left(\frac{\mu}{\rho}\right)_{x_{ij}} - \left(\frac{\mu}{\rho}\right)_{\text{prot}_j}}{\left(\frac{\mu}{\rho}\right)_{\text{prot}_j}} \right| \dots\dots\dots (55).$$

In biologic material practically all of the more common elements have atomic numbers (Z) lower than 30. For these elements equation (54) is diagrammatically represented in two different ways.

For monochromatic roentgen radiation at six different wavelengths between 2.5 and 22 kX.U., the relative amount of different elements is plotted as a function of the atomic number in Fig. 17 a—f. The axis of ordinates has a logarithmic scale. *The partial systematic errors ε_{ij} are fixed at ± 5 per cent*, where the positive sign is used when $\left(\frac{\mu}{\rho}\right)_{x_{ij}} > \left(\frac{\mu}{\rho}\right)_{\text{prot}_j}$, and the negative sign when $\left(\frac{\mu}{\rho}\right)_{x_{ij}} < \left(\frac{\mu}{\rho}\right)_{\text{prot}_j}$. As ε_{ij} is proportional to the relative amount of x_i , the shape of the curves is independent of the absolute value of ε_{ij} . Where the differential coefficient (derivative) of the curves is negative, the systematic error is positive, and *vice versa*. All the curves have a maximum in the neighbourhood of $Z=7$, depending upon the small difference between the mass absorption coefficients of an average animal protein and nitrogen.

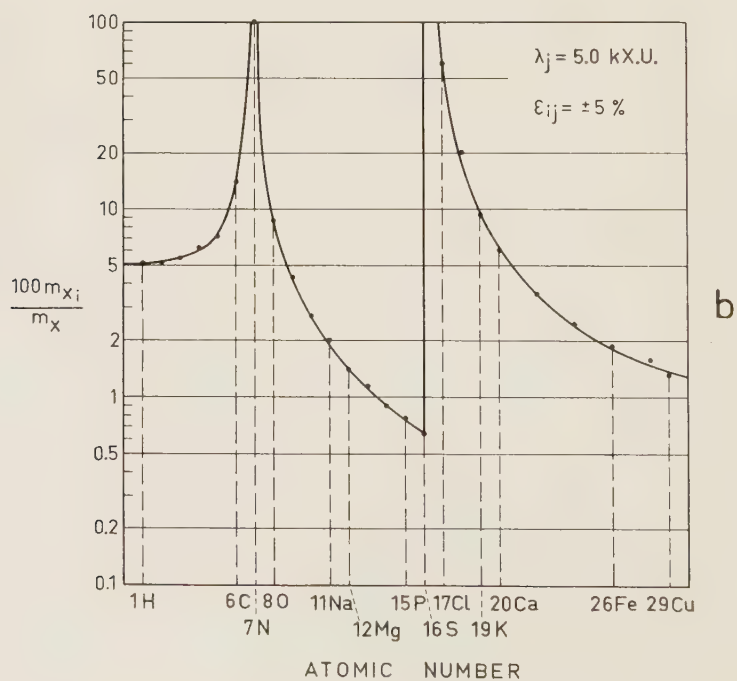
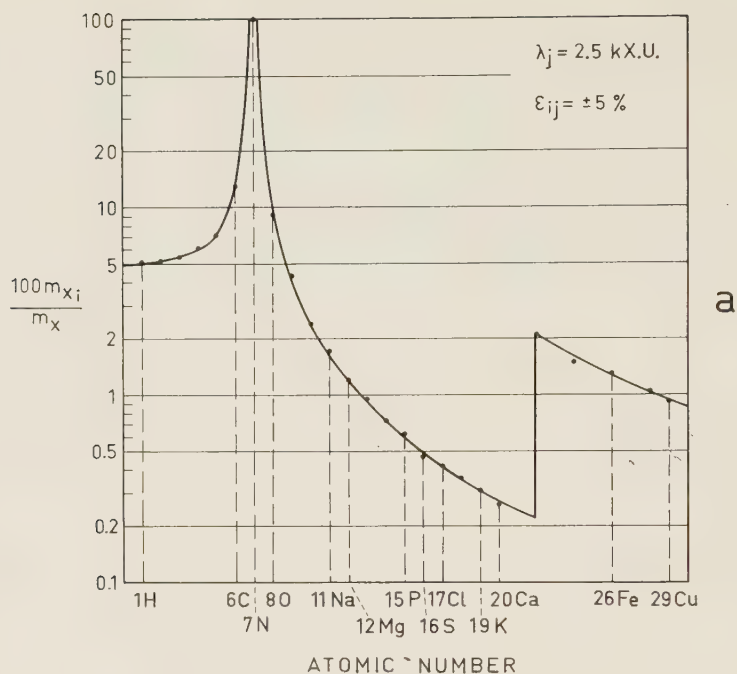


Fig. 17 a—b. The relative amounts of different elements as a function of the atomic number.

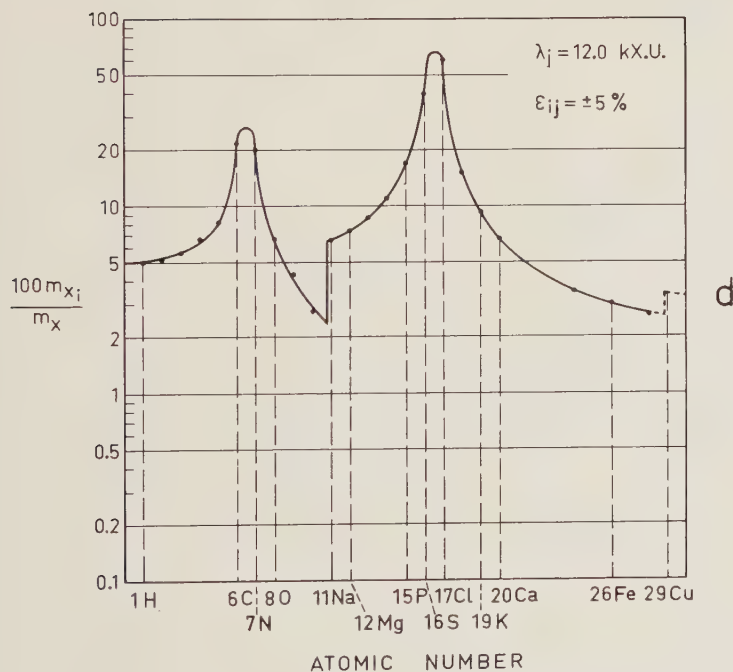
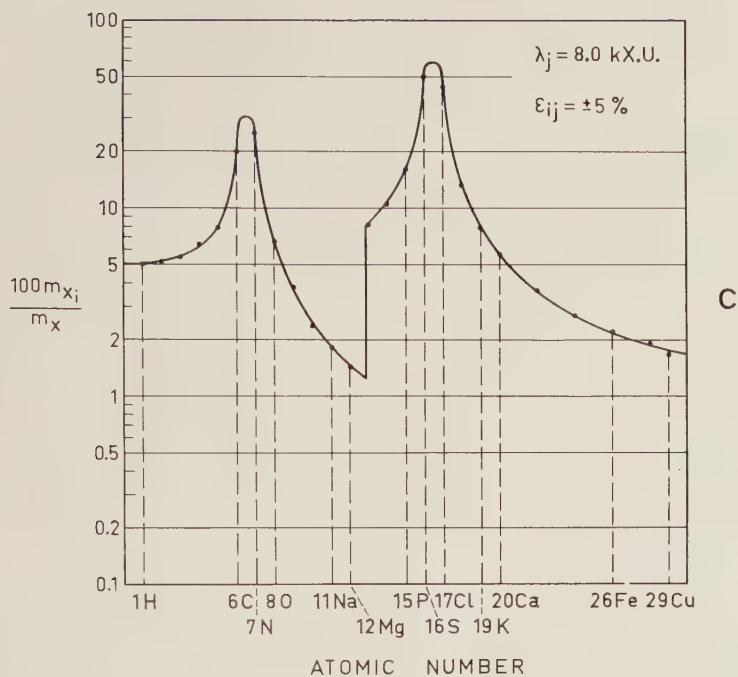


Fig. 17 c—d. The relative amounts of different elements as a function of the atomic number.

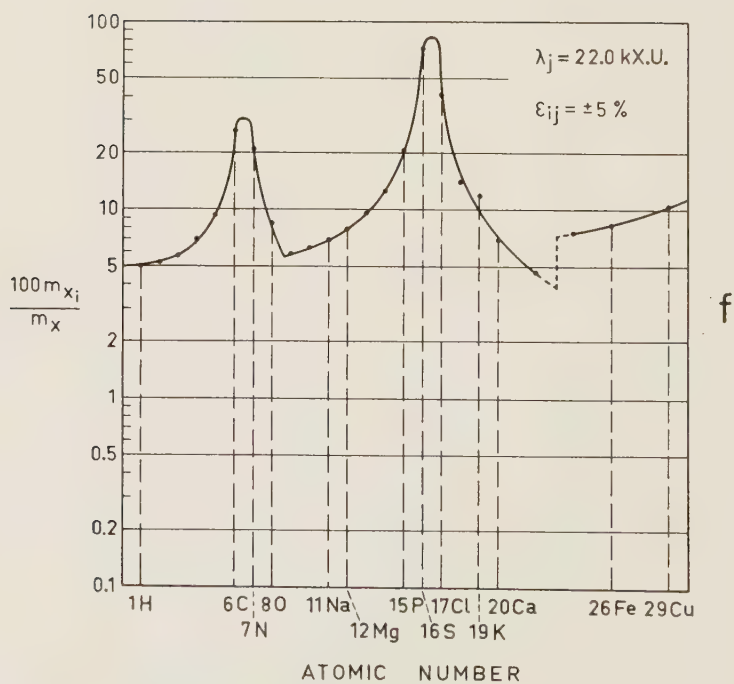
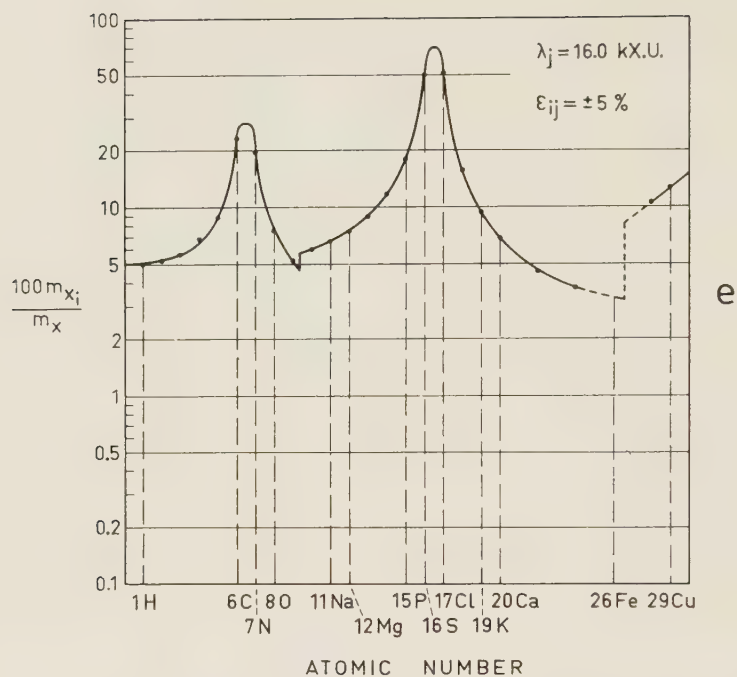


Fig. 17 e—f. The relative amounts of different elements as a function of the atomic number.

In fact, the partial systematic error for nitrogen cannot amount to ± 5 per cent at wavelengths between 2.5 and 5.0 kX.U. From $Z=1$ to $Z=7$ the shape of the curves does not change with the wavelength of the roentgen radiation because of the constant ratios between the corresponding mass absorption coefficients.

The discontinuity between $Z=21$ and $Z=22$ in the curve in Fig. 17 a depends on the fact that the K absorption limits of the elements with $Z \geq 22$ are situated at wavelengths shorter than 2.5 kX.U. The corresponding discontinuity in the curve in Fig. 17 b is situated between sulphur (K absorption limit at 5.01 kX.U.) and chlorine (K absorption limit at 4.38 kX.U.). The curves in Fig. 17 c—f have the same general shape with a second maximum corresponding to the elements phosphorus, sulphur, and chlorine. Towards longer wavelengths an increasing number of elements with $Z \leq 16$ have negative systematic errors, according as the wavelength of the roentgen radiation is longer than that of the K absorption limit of the different elements. The dash part of each curve in Fig. 17 d—f corresponds to elements with L absorption limits at the wavelength in question. As accurate values of the mass absorption coefficients between the L_I and L_{III} absorption limits could not be obtained, the shape of the curves is only approximate in these regions.

In Fig. 18 a—b the partial systematic errors of the more common elements in biologic material are plotted as a function of the wavelength. *The relative amount of each element is fixed at 10 per cent.* As, according to equation (54), the partial systematic error ε_{ij} is proportional to the relative amount of the element x_i , the errors at other concentrations can also be obtained from the curves. In Fig. 18 b the scale of the axis of ordinates is one fourth of that in Fig. 18 a.

The curves in Fig. 18 a—b show that the systematic error is generally smaller at longer wavelengths. The curves have a discontinuity at the K absorption limit of sulphur at 5.01 kX.U., as the mass absorption coefficient of an average animal protein has also a discontinuity at that wavelength because of the content of sulphur in protein.

With regard to the shape of the curves the elements can be divided into three groups. The *first* group includes *hydrogen, carbon, nitrogen, and oxygen*, the systematic errors of which are approximately constant in the whole wavelength region. The *second* group comprises *sodium, magnesium, phosphorus, sulphur, chlorine, potassium, and calcium*, which have their K

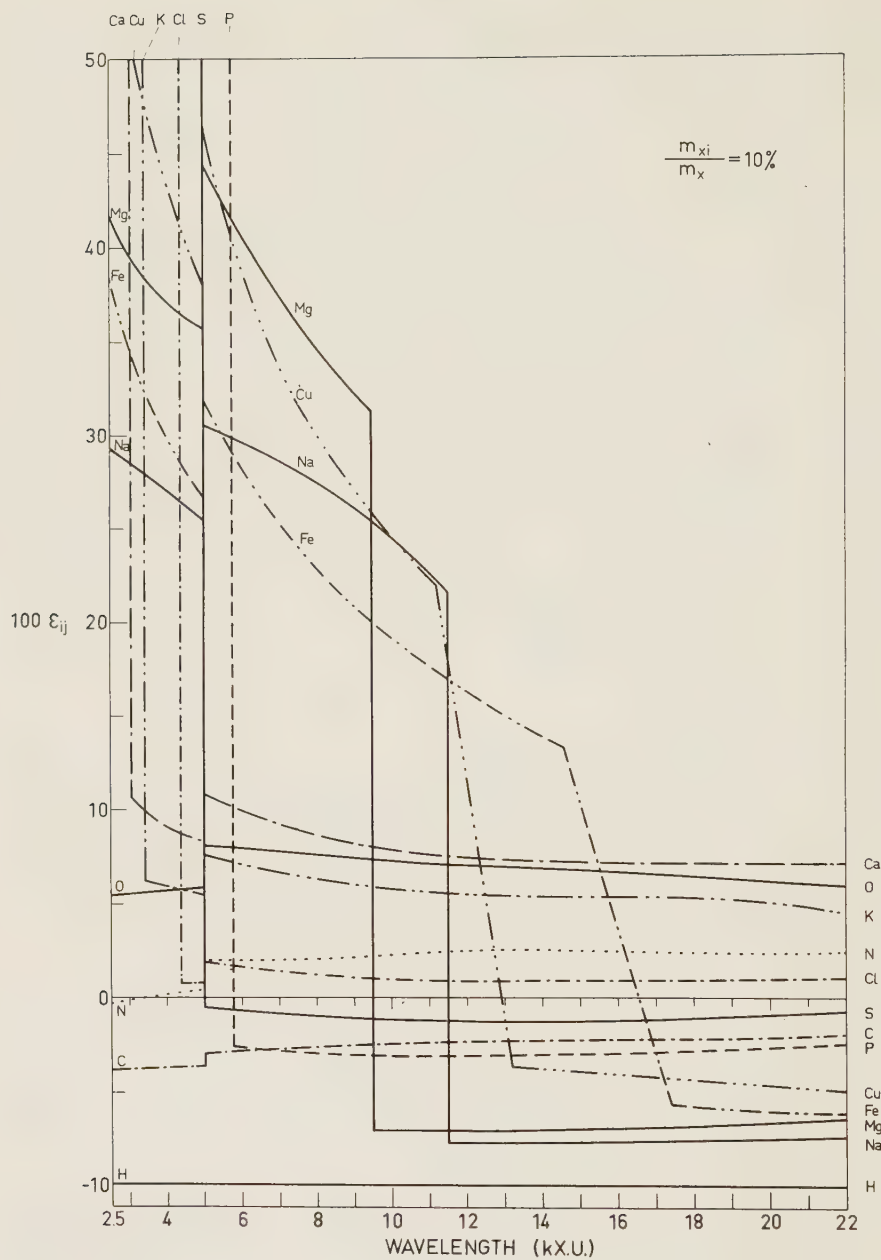


Fig. 18 a. The partial systematic errors ϵ_{ij} of common elements in biologic material as a function of the wavelength.

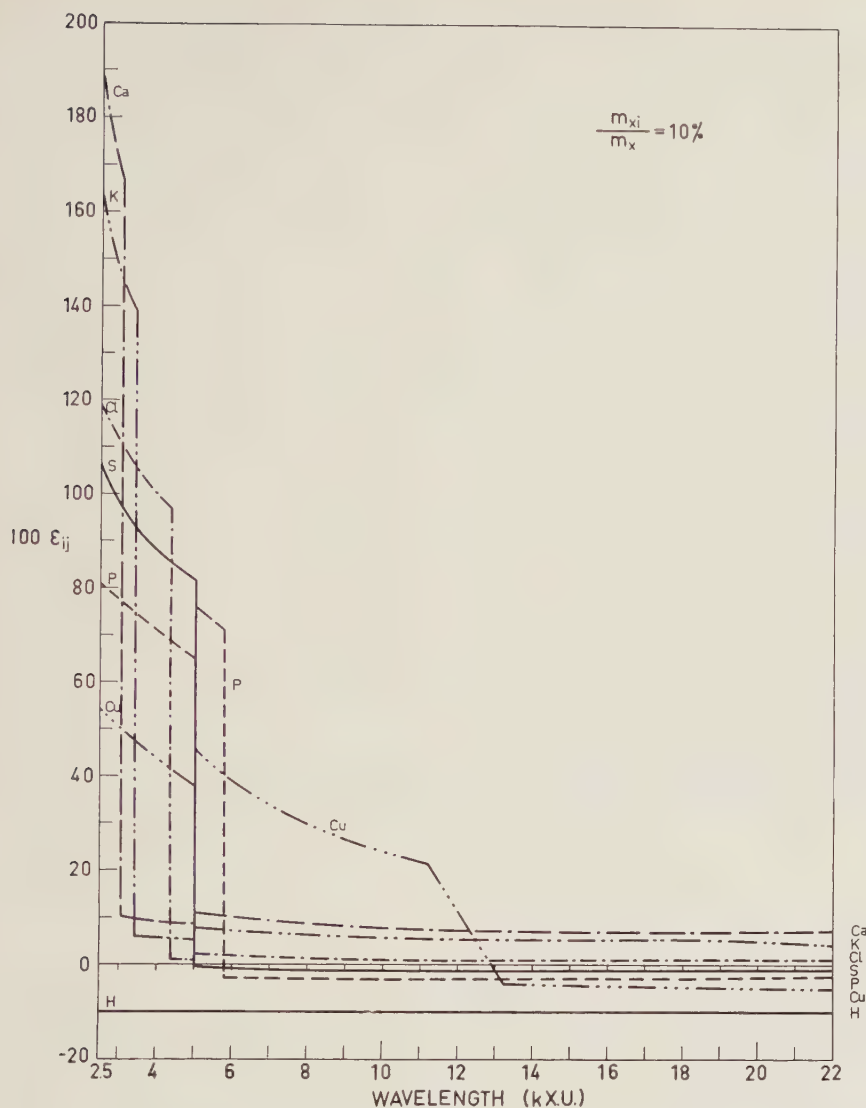


Fig. 18 b. The partial systematic errors ϵ_{ij} of common elements in biologic material as a function of the wavelength.

absorption limits in the wavelength range between 2.5 and 22 kX.U. A discontinuous decrease of the systematic error ϵ_{ij} of each of these elements occurs at the wavelength corresponding to the K absorption limit in question. For phosphorus the systematic error is about 70 per cent on the short wave-

length side of the K absorption limit at 5.77 kX.U. and about -2.5 per cent on the long wavelength side. For sulphur the corresponding values are 82 per cent and -0.5 per cent at the K absorption limit of this element at 5.01 kX.U. On the short wavelength side of each limit the systematic error ε_{ij} of the element in question increases continuously towards shorter wavelengths, but on the long wavelength side of the limit the systematic error is approximately constant.

Iron and copper, constituting the *third* group, have L absorption limits in the wavelength region to be utilized for total dry weight determination of biologic material. The influence of these discontinuities on the systematic errors is in principle the same as that of the K absorption limit of the elements in the second group. Between the L_I and L_{III} absorption limits no accurate values of the mass absorption coefficients could be obtained. Therefore, a straight line connects the beginning of the L_I discontinuity and the end of the L_{III} discontinuity on the error curve instead of three separate discontinuities, corresponding to the three L absorption limits of each element.

The change of the sign of ε_{ij} at the discontinuity of the corresponding error curve characterizes the curves for sodium, magnesium, phosphorus, sulphur, iron, and copper. Hydrogen and carbon have negative systematic errors in the whole wavelength region, whereas nitrogen (with the exception of a small region from 2.5 to 3.5 kX.U.), oxygen, chlorine, potassium, and calcium have positive systematic errors. Therefore, the partial systematic errors of the different elements can compensate each other to a certain extent at wavelengths longer than about 6 kX.U., if several of these elements are mixed with protein, as is generally the case in biologic material.

Equation (54) is also valid for different organic compounds x_i , mixed with the average animal protein. Mass absorption coefficients of some special kinds of proteins, of desoxyribonucleic acid (DNA) and ribonucleic acid (RNA), of glycogen, and of some common lipids, presented in Table VII, have been calculated to one figure more than in Table IV. These organic compounds are composed of the same elements with few minor exceptions. Therefore, the uncertainty in the last figure will have a very small influence upon the value of the ratio $\left(\frac{\mu}{\rho}\right)_{ij} / \left(\frac{\mu}{\rho}\right)_{\text{prot } j}$ when determining ε_{ij} , but with this extra figure the continuity of the values of ε_{ij} at successive wavelengths is improved.

In Fig. 19 the partial systematic errors ε_{ij} of the different organic com-

$$\frac{m_{xi}}{m_x} = 10\%$$

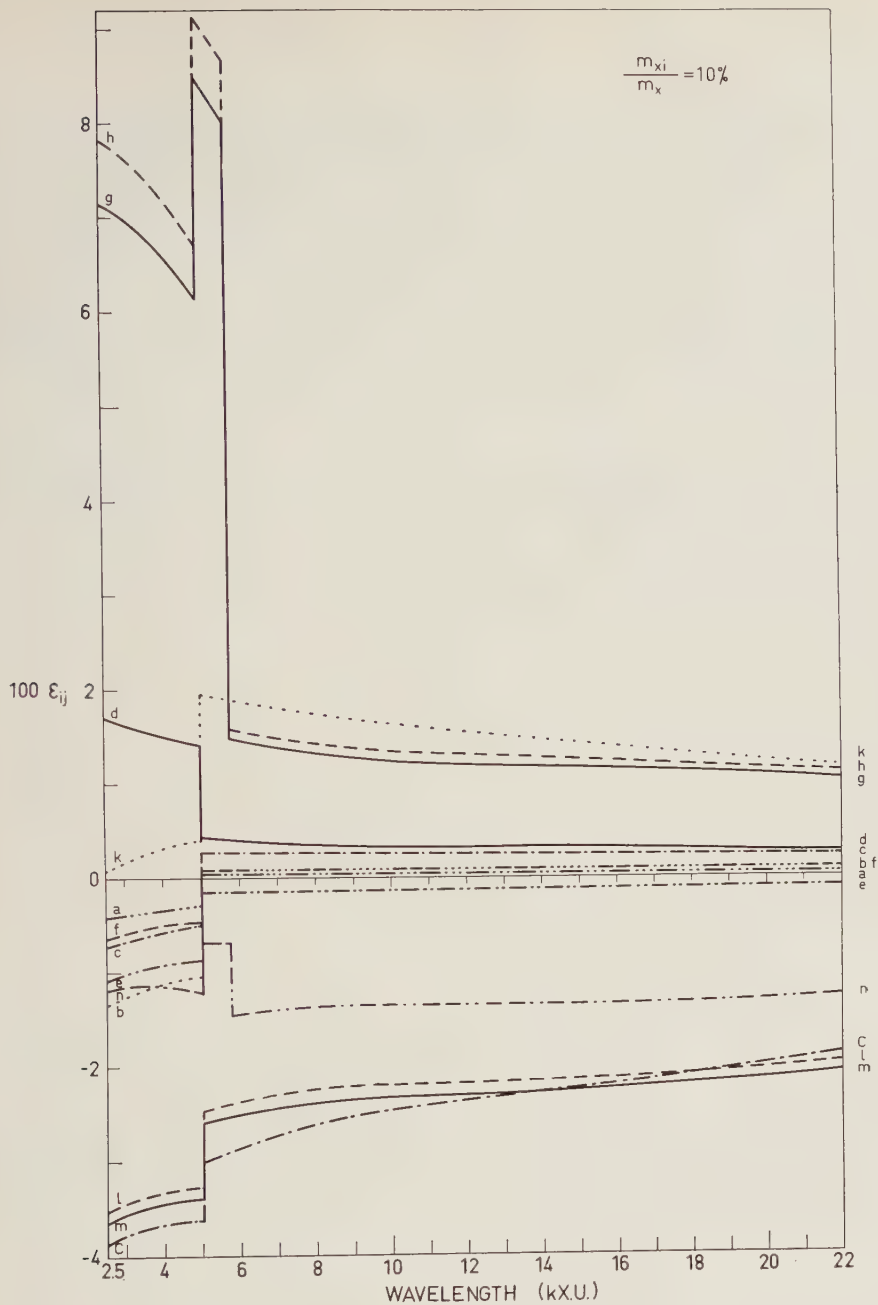


Fig. 19. The partial systematic errors ϵ_{ij} of some common organic compounds as a function of the wavelength.

- | | | |
|------------|--------------------------|----------------|
| a Fibrin | e Thymus histone | k Glycogen |
| b Elastin | f Plant proteins | l Tripalmitin |
| c Collagen | g Desoxyribonucleic acid | m Tristearin |
| d Amyloid | h Ribonucleic acid | n Cerebrosides |

pounds are plotted as functions of the wavelength of the roentgen radiation. The relative amount of each compound is fixed at 10 per cent. These curves are the counterparts to the curves in Fig. 18 a—b, but the scale of the axis of ordinates is five times greater than that in Fig. 18 a. In comparison the error curve of carbon is also included in the diagram.

All the curves in Fig. 19 have a discontinuity at 5.01 kX.U., corresponding to the K absorption limit of sulphur. The curves of RNA, DNA, and cerebro-sides have a second discontinuity at the K absorption limit of phosphorus at 5.77 kX.U. On the long wavelength side of these discontinuities the absolute values of ϵ_{ij} are generally smaller. However, cerebro-sides have an approximately constant systematic error in the whole wavelength region, and the systematic error of glycogen is 0.4 per cent on the short wavelength side and 1.9 per cent on the long wavelength side of the discontinuity.

With regard to the magnitude of the systematic errors ϵ_{ij} at wavelengths longer than those of the discontinuities, the curves can be divided into three groups. The *first* group includes *glycogen*, *RNA*, and *DNA* which have positive systematic errors, varying between 1 and 2 per cent. The *second* group comprises *the different proteins*, i.e. amyloid, collagen, elastin, average plant protein, fibrin, and thymus histone. In this group the partial systematic errors are between -0.2 and 0.4 per cent at wavelengths longer than 5.01 kX.U. The error curves of the average plant protein and elastin coincide in this wavelength region. At shorter wavelengths the systematic errors are between -1.4 and 1.7 per cent, depending upon the varying concentration of sulphur in the different proteins.

The *third* group includes the error curves of the *lipids*. The systematic error of cerebro-sides varies between -1.5 and -1.3 per cent at wavelengths longer than 5.01 kX.U. In the same wavelength range the partial systematic errors of the most common triglycerides are between -2.6 and -2.0 per cent. The sequence between the different triglycerides is tripalmitin, trilinolein, triolein, and tristearin, where tripalmitin has the smallest systematic error and tristearin the greatest one. Only the error curves of tripalmitin and tristearin are represented in Fig. 19. The difference between the ordinates of these two curves is small and varies between 0.10 and 0.15 pr cent at different wavelengths.

C. The Systematic Errors Utilizing a Continuous Roentgen Spectrum

In the preceding section the partial systematic errors were determined when monochromatic roentgen radiation with the wavelength λ_j was utilized. *Soft* roentgen radiation is used in the quantitative microradiographic procedure for total dry weight determinations of small biologic objects in order to obtain a high contrast in the roentgen absorption image. Moreover, *high* intensities are necessary, as the fine-grained photographic emulsions are extremely insensitive [143]. Therefore, *soft, continuous* roentgen radiation must be utilized.

The total dry weight determinations to be described have been performed at a voltage across the roentgen tube of 1.5 kV or lower. As shown in the preceding section, the partial systematic errors ε_{ij} generally decrease at longer wavelengths. The resulting systematic errors are therefore calculated for the most unfavourable case, i.e. at a voltage of 1.5 kV.

The shape of a continuous roentgen spectrum generated at low voltages approaches that obtained from a thin target, as discussed in detail in Chapter 1: A (see Fig. 1). From different investigations of soft continuous roentgen spectra expressions (12) and (15) have been found to satisfy equation (8). Therefore, the approximate distribution of the intensity in the utilized, continuous roentgen spectrum has been calculated from the two expressions (12) and (15). The continuous roentgen spectrum is filtered through two aluminium foils with a total weight of $0.33 \text{ mg} \cdot \text{cm}^{-2}$ and through a supporting zapon varnish foil on the metal disc (see Fig. 16) with an approximate weight of $0.2 \text{ mg} \cdot \text{cm}^{-2}$. The effect of filtration has been calculated according to equation (23), and the mass absorption coefficients have been taken from Table IV and Table XI respectively. The intensity distribution in the *filtered*, continuous roentgen spectrum is shown in Fig. 20 a—b, and the relative distribution of the intensity is given in Table VIII.

When utilizing the filtered, continuous roentgen spectrum, the resulting systematic error ε of n elements or compounds x_i is determined in the following way. In equations (49) and (50) $m_{\alpha j}$ is eliminated, which gives

$$I_{1j} = I_{0j} e^{-\left(\frac{\mu}{\rho}\right)_{\text{prot}j} (1 + \varepsilon_j) m_x} \dots \dots \dots (56).$$

The continuous roentgen spectrum has been divided in n_1 regions, each

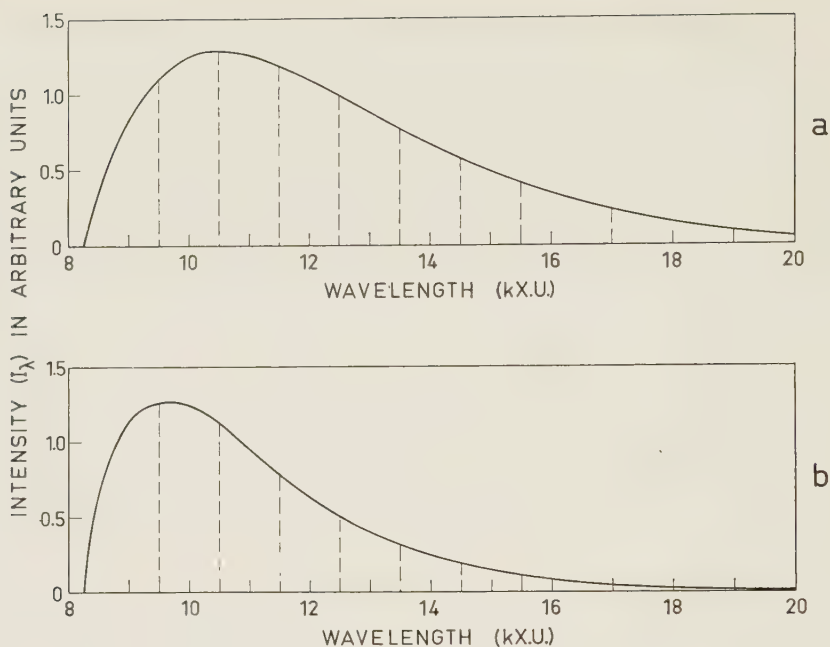


Fig. 20 a—b. The intensity distribution in the filtered, continuous roentgen spectrum generated at 1.5 kV. a) Calculated according to equation (12). b) Calculated according to equation (15).

with a mean wavelength λ_j . The intensities in the different regions are added according to

$$\sum_{j=1}^{n_1} I_{1j} = \sum_{j=1}^{n_1} I_{0j} e^{-\left(\frac{\mu}{\rho}\right)_{\text{prot}_j} (1 + \varepsilon_j) m_x} = \sum_{j=1}^{n_1} I_{0j} e^{-\left(\frac{\mu}{\rho}\right)_{\text{prot}_j} (1 + \varepsilon) m_x} \quad \dots \quad (57),$$

where ε is the resulting systematic error.

When performing the averaging procedure the exponential expressions are developed according to

$$e^x = 1 + x + \frac{x^2}{2!} + \frac{x^3}{3!} + \dots \quad (58).$$

As m_x is generally very small, square and higher powers can be neglected. As this approximation is introduced on both sides of equation (57), the resultant error is small. Hence

Table VIII. The intensity distribution in the filtered, continuous roentgen spectrum, generated at 1.5 kV

Wavelength region kX.U.	Distribution of intensity in per cent, according to	
	equation (12) Fig. 20 a	equation (15) Fig. 20 b
8.25 — 9.5	11.5	23.8
9.5 — 10.5	16.7	24.8
10.5 — 11.5	17.0	19.2
11.5 — 12.5	14.8	12.8
12.5 — 13.5	11.9	8.1
13.5 — 14.5	9.1	4.9
14.5 — 15.5	6.6	2.9
15.5 — 17.0	6.4	2.2
17.0 — 19.0	4.3	1.0
19.0 — ∞	1.7	0.3

$$\sum_{j=1}^{n_1} I_{0j} \left[1 - \left(\frac{\mu}{\rho} \right)_{\text{prot}_j} (1 + \varepsilon_j) m_x \right] = \sum_{j=1}^{n_1} I_{0j} \left[1 - \left(\frac{\mu}{\rho} \right)_{\text{prot}_j} (1 + \varepsilon) m_x \right] \dots \quad (59).$$

After transformations the following is obtained:

$$m_x \sum_{j=1}^{n_1} I_{0j} \left(\frac{\mu}{\rho} \right)_{\text{prot}_j} \varepsilon_j = m_x \varepsilon \sum_{j=1}^{n_1} I_{0j} \left(\frac{\mu}{\rho} \right)_{\text{prot}_j} \dots \dots \dots (59 \text{ a}).$$

Equation (59 a) solved for ε , gives

$$\varepsilon = \frac{\sum_{j=1}^{n_1} I_{0j} \left(\frac{\mu}{\rho} \right)_{\text{prot}_j} \varepsilon_j}{\sum_{j=1}^{n_1} I_{0j} \left(\frac{\mu}{\rho} \right)_{\text{prot}_j}} \dots \dots \dots (60).$$

Table IX. The resulting systematic error ε_i of different elements and organic compounds for the filtered, continuous roentgen spectrum, generated at 1.5 kV. The relative amount of each element 10 per cent

Element	100 ε_i Intensity distribution according to		Compound	100 ε_i Intensity distribution according to	
	Fig. 20 a	Fig. 20 b		Fig. 20 a	Fig. 20 b
1 H	— 9.9	— 9.9	Fibrin	0.03	0.03
6 C	— 2.3	— 2.4	Elastin	0.06	0.06
7 N	2.4	2.4	Collagen	0.26	0.26
8 O	6.9	7.1	Amyloid	0.32	0.32
11 Na	~ 0.2	~ 7.7	Thymus histone	— 0.12	— 0.12
12 Mg	~ — 5.1	~ — 1.9	Plant protein	0.05	0.05
15 P	— 2.9	— 3.0	DNA	1.19	1.22
16 S	— 1.1	— 1.1	RNA	1.26	1.29
17 Cl	0.9	0.9	Glycogen	1.47	1.52
19 K	5.4	5.5	Tripalmitin	— 2.17	— 2.19
20 Ca	7.3	7.5	Tristearin	— 2.29	— 2.32
26 Fe	~ 12.0	~ 15.8	Triolein	— 2.23	— 2.26
29 Cu	~ 8.8	~ 16.0	Trilinolein	— 2.18	— 2.21
			Cerebrosides	— 1.37	— 1.37

If ε_j is constant, the equality $\varepsilon = \varepsilon_j$ is immediately obtained. For a single element x_i , i.e. for $n=1$, equation (60) is also valid, if ε_i and ε_{ij} are substituted for ε and ε_j respectively. In this equation I_{0j} can be replaced by the corresponding percentage in Table VIII, and $\left(\frac{\mu}{\rho}\right)_{\text{prot } j}$ is obtained from Table VII. When the relative amount of the element or compound x_i is 10 per cent, ε_{ij} is obtained from Fig. 18 a—b and Fig. 19 respectively.

The resulting systematic errors ε_i of individual elements and compounds have been determined from equation (60) and are presented in Table IX. The two values for each element and compound correspond to the two different distributions of the intensity of the roentgen radiation according to Fig. 20 a and b respectively. For the organic compounds and for most of the elements in Table IX the difference between the two values is small. Because of absorption discontinuities and a change of the sign of the partial systematic

errors ε_{ij} in the wavelength range from 8 to 20 kX.U., the corresponding values of sodium, magnesium, iron, and copper are more divergent. As only relatively low concentrations of these elements occur in biologic material, the uncertainty of the resulting systematic errors ε_i is insignificant, although the exact shape of the filtered, continuous roentgen spectrum has not been determined. The filtration of the roentgen radiation in the specimen and differences in the photographic response to constant intensities of roentgen radiation with different wavelengths will have a negligible influence upon the calculated values of ε_i in Table IX.

The *common* elements in biologic material with positive and negative systematic errors, respectively, are about equal in number, and therefore the systematic errors of the different elements compensate each other to a considerable extent. Iron, copper, and other elements with higher atomic numbers are present in very small concentrations in biologic material. The amount of calcium in calcified tissues, however, involves a considerable error [30]. Therefore, quantitative microradiographic determinations of the total dry weight cannot be performed on calcified tissues, unless the amount of calcium is determined by means of an independent method.

The resulting systematic errors of *the different proteins* are negligible. Utilizing roentgen radiation in this wavelength region, the positive systematic errors of RNA and DNA are caused by the high percentage of oxygen and *not* by the high relative amount of phosphorus in these compounds. However, 10 per cent RNA or DNA gives a resulting systematic error of only about 1.25 per cent. The same relative amount of *glycogen* involves a systematic error of 1.5 per cent.

The *lipids* have negative resulting systematic errors because of the high percentages of carbon and hydrogen in these compounds. For a mixture of 90 per cent protein and 10 per cent lipids the resulting systematic error is about -2.2 per cent. It may therefore be necessary to introduce a correction factor, when the microradiographic procedure is applied to tissues with high concentrations of lipids. This is especially required, when the lipid fraction is determined by total dry weight determinations before and after lipid extraction. *If no such correction is made, the error of the weight of the lipid fraction will be of the magnitude of between -20 and -25 per cent, i.e. the determined weight will be about 75 to 80 per cent of the correct dry weight.*

D. The Reference System and the Final Calculation of Weight per Unit Area

As described in Chapter 5:C and schematically illustrated in Fig. 16, the total dry weight of a biologic structure is determined by comparing the photo-current corresponding to the roentgen absorption image of the structure with those corresponding to the image of the step wedge of nitrocellulose foils. By means of the calibration curve (the photo-current versus the number of foils) the photometer deflections for the structures are transformed to foil equivalents (u_1 and u_2 respectively). In this way the weight per unit area of a reference system with the same absorption as that of the biologic structure is determined.

For a continuous roentgen spectrum this principle of comparison can be expressed by the following equation

$$\sum_{j=1}^{n_1} I_{1j} = \sum_{j=1}^{n_1} I_{0j} e^{-\left(\frac{\mu}{\rho}\right)_{\text{prot}_j} (1+\varepsilon) m_x} = \sum_{j=1}^{n_1} I_{0j} e^{-\left(\frac{\mu}{\rho}\right)_{\text{ref}_j} m_{\text{ref}}} \dots\dots (61),$$

analogous to equation (57). Here $\left(\frac{\mu}{\rho}\right)_{\text{ref}_j}$ is the mass absorption coefficient of the reference system at the wavelength λ_j , and m_{ref} is given by

$$m_{\text{ref}} = u_x w \dots\dots\dots (62),$$

where u_x is the foil equivalent of the biologic structure, and w is the weight per unit area of one foil in the reference system. Equation (61) can be transformed to the following expression

$$\sum_{j=1}^{n_1} I_{0j} \left(\frac{\mu}{\rho}\right)_{\text{prot}_j} (1+\varepsilon) m_x = \sum_{j=1}^{n_1} I_{0j} \left(\frac{\mu}{\rho}\right)_{\text{ref}_j} m_{\text{ref}} \dots\dots\dots (63),$$

which is analogous to equation (59 a), and can be solved for the weight per unit area of the biologic structure:

$$(1+\varepsilon) m_x = m_{\text{ref}} \frac{\sum_{j=1}^{n_1} I_{0j} \left(\frac{\mu}{\rho}\right)_{\text{ref}_j}}{\sum_{j=1}^{n_1} I_{0j} \left(\frac{\mu}{\rho}\right)_{\text{prot}_j}} \dots\dots\dots (64).$$

Table X. The elementary composition of some different cellulose nitrates

Compound	% C	% H	% N	% O
Parlodion	28.1	3.6	11.5	56.8
Zapon varnish	46.7	5.3	6.6	41.4
Cellulose dinitrate	34.79	4.38	6.76	54.07
Cellulose trinitrate	31.38	3.73	9.15	55.74
Cellulose tetranitrate	28.58	3.20	11.11	57.11
Cellulose pentanitate	26.24	2.75	12.75	58.26
Cellulose hexanitate	24.25	2.38	14.14	59.23

If $\left(\frac{\mu}{\rho}\right)_{\text{ref}_j} / \left(\frac{\mu}{\rho}\right)_{\text{prot}_j}$ is constant at different wavelengths, equation (64) is simplified to

$$(1 + \varepsilon) m_x = m_{\text{ref}} \frac{\left(\frac{\mu}{\rho}\right)_{\text{ref}_j}}{\left(\frac{\mu}{\rho}\right)_{\text{prot}_j}} \dots \dots \dots (64 \text{ a}).$$

This is the reason why the reference system ought to have about the same elementary composition or at least be composed of the same elements as the average protein.

The elementary compositions of the Parlodion foils, used as reference system, of the zapon varnish foils, used as supporting foils for the specimens, and of some other cellulose nitrates are given in Table X. Mass absorption coefficients of these compounds are presented in Table XI. For a reference system, composed of Parlodion foils, the ratio between $\left(\frac{\mu}{\rho}\right)_{\text{ref}_j}$ and $\left(\frac{\mu}{\rho}\right)_{\text{prot}_j}$ at different wavelengths is given in Table XII. The ratio is approximately constant at wavelengths longer than 6.0 kX.U., but a continuous, small decrease of the ratio is obtained at increasing wavelengths. With the distribution of the intensity of the roentgen radiation according to Fig. 20 a, equation (64) takes the form

Table XI. Mass absorption coefficients

Compound	2.5 kX.U.	3.0 kX.U.	3.5 kX.U.	4.0 kX.U.	5.0 kX.U.	6.0 kX.U.	7.0 kX.U.	8.0 kX.U.
Parlodion	36.1	63.5	100.6	151.1	288	477	745	1074
Zapon varnish	30.8	54.3	86.0	128.8	245	405	636	924
Cellulose dinitrate	34.6	60.9	96.6	144.8	276	456	714	1031
Cellulose trinitrate	35.5	62.5	99.0	148.5	283	468	732	1057
Cellulose tetranitrate	36.2	63.7	101.0	151.6	289	478	747	1078
Cellulose pentanitate	36.8	64.8	102.6	154.1	293	486	760	1096
Cellulose hexanitate	37.3	65.7	104.1	156.3	298	493	771	1111

Table XII. The ratio between the mass absorption coefficients of

Ratio	2.5 kX.U.	3.0 kX.U.	3.5 kX.U.	4.0 kX.U.	5.0 kX.U.	6.0 kX.U.	7.0 kX.U.	8.0 kX.U.
$\left(\frac{\mu}{\rho}\right)_{\text{ref}_j} / \left(\frac{\mu}{\rho}\right)_{\text{prot}_j}$	1.157	1.167	1.174	1.187	1.200	1.375	1.362	1.343

$$(1 + \varepsilon)m_x = 1.315 \, m_{\text{ref}} \quad \dots\dots\dots (64 \, b),$$

and according to Fig. 20 b the following form:

$$(1 + \varepsilon)m_x = 1.323 \, m_{\text{ref}} \quad \dots\dots\dots (64 \, c).$$

The mean value 1.32 has been used in the following calculations.

When the utilized, extremely soft roentgen spectrum is generated by a continuously evacuated roentgen tube, the introduction of a reference system has three main advantages.

1) The principle of comparison makes it unnecessary to determine the density of the fine-grained photographic emulsion as a function of the intensity of the roentgen radiation.

2) The photographic effect of the extremely soft, continuous roentgen spectrum transmitted through the specimen may not be the same as that of an incident continuous roentgen spectrum with the same intensity, because of the filtration of the extremely soft roentgen spectrum in the specimen. This

of some cellulose nitrates

9.0 kX.U.	10.0 kX.U.	11.0 kX.U.	12.0 kX.U.	13.0 kX.U.	14.0 kX.U.	15.0 kX.U.	16.0 kX.U.	18.0 kX.U.	20.0 kX.U.	22.0 kX.U.
1491	1944	2560	3250	3980	4830	5790	6690	9090	11820	14810
1280	1679	2210	2800	3440	4170	5020	5810	7910	10330	13010
1429	1865	2460	3120	3820	4630	5560	6420	8730	11360	14250
1466	1912	2520	3200	3910	4750	5700	6580	8940	11630	14580
1495	1950	2570	3260	3990	4840	5810	6710	9120	11830	14830
1520	1983	2610	3320	4060	4920	5910	6820	9270	12040	15080
1541	2010	2650	3360	4120	4990	5990	6910	9390	12200	15280

parlodion and the average animal protein at different wavelengths

9.0 kX.U.	10.0 kX.U.	11.0 kX.U.	12.0 kX.U.	13.0 kX.U.	14.0 kX.U.	15.0 kX.U.	16.0 kX.U.	18.0 kX.U.	20.0 kX.U.	22.0 kX.U.
1,342	1,322	1,325	1,332	1,318	1,320	1,310	1,302	1,293	1,288	1,272

possible source of error is eliminated when utilizing a system of comparison, as the filtration of the continuous roentgen spectrum in the specimen is about the same as that in the reference system with the same total absorption.

3) The exact value of the resulting mass absorption coefficient of the average animal protein is not necessary to determine, which is important since the utilized thin filters must be changed very often and have not exactly the same thickness. Moreover, a possible effect of filtration in the specimen will change the value of the mass absorption coefficient.

CHAPTER 7

The Experimental Apparatus

The roentgen tube utilized for total dry weight determinations is of essentially the same construction as the tube designed for the high vacuum roentgen ray spectrograph to be described in Part III. As originally planned, the roentgen tube for total dry weight determinations can therefore also be utilized as the roentgen source for a roentgen ray spectrograph. The roentgen tube was constructed to make it possible to obtain extremely soft roentgen radiation with a high intensity.

A. The Roentgen Tube

The constructional drawings of the roentgen tube are presented in Fig. 21—23. The grounded central part (1) of the roentgen tube is made of forged brass and is water-cooled through a drilled loop (1 a). By means of a porcelain insulator (2) the anode (3 a—b) is insulated from (1). In most of the vacuum connections Gaco O-rings (type P 60, G. Angus and Co. Ltd., Newcastle upon Tyne) are used. The axis of the anode is horizontal and at right angles to the axis of the cathode (4). These two axes are situated in the same vertical plane as the central axis of the specimen holder (5), which forms an angle of 8° with the axis of the anode. The metal tube (6) in Fig. 23 is connected to the vacuum pumps by means of a tombak tube, and to the space inside the roentgen tube by the drilled hole (6 a) in Fig. 22. As the cross sections through (6) and through the remaining part of the roentgen tube are made in different planes in Fig. 23, the connection (6 a) is not seen in this figure.

The inner part (3 a) of the *anode* consists of copper, which has a high thermal conductivity. On the cylindrical envelope surface of (3 a) twelve grooves (7) with flanges are cut, into which different anode materials (8), in the form of plates $1 \times 8 \times 50$ mm, can be tapped. According to equation (1) the efficiency of a roentgen tube is proportional to the atomic number Z of the anode material, and according to equation (6) the total energy of a continuous roentgen spectrum is also proportional to Z . Therefore, platinum

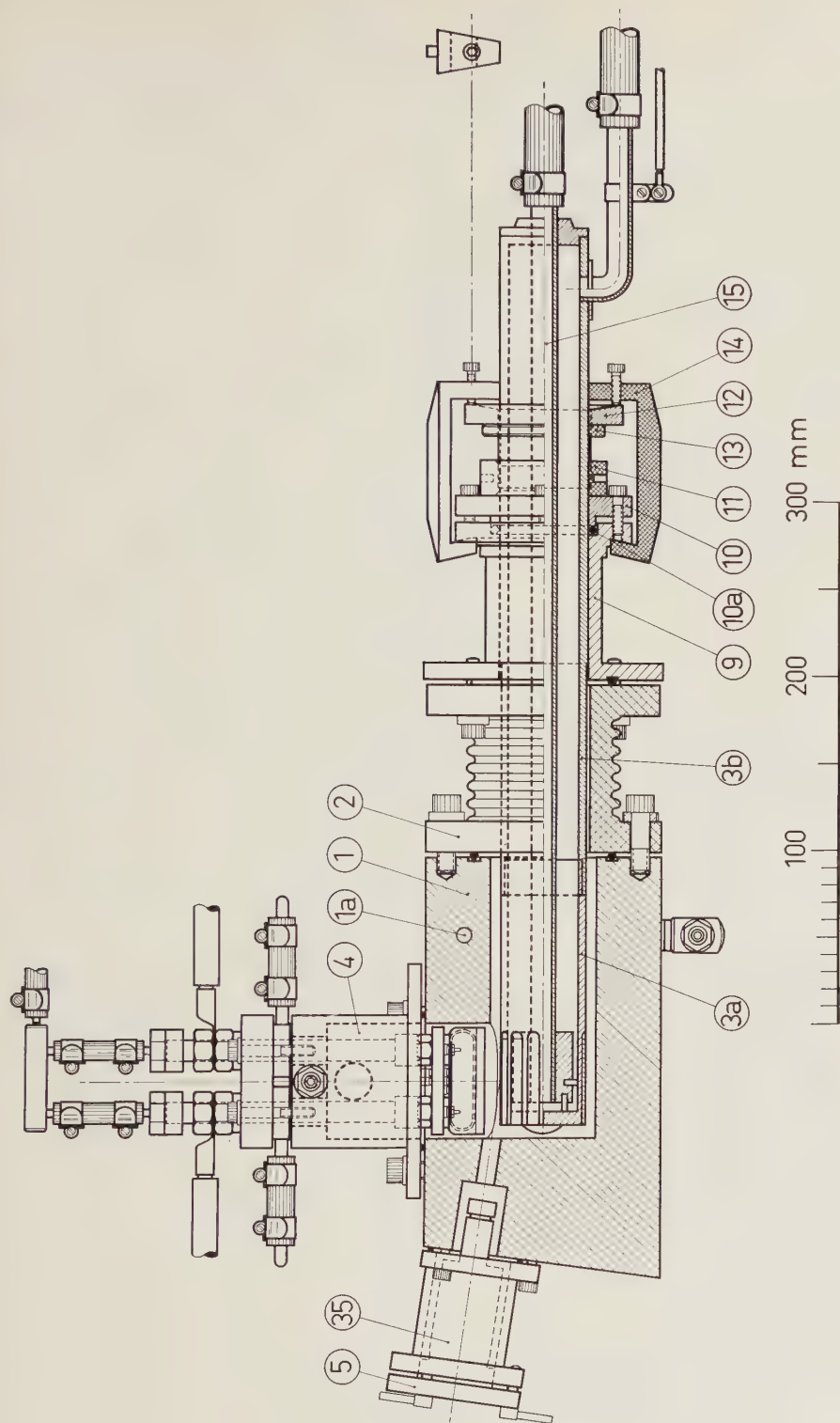


Fig. 21. Longitudinal section through the anode and the central part of the roentgen tube.

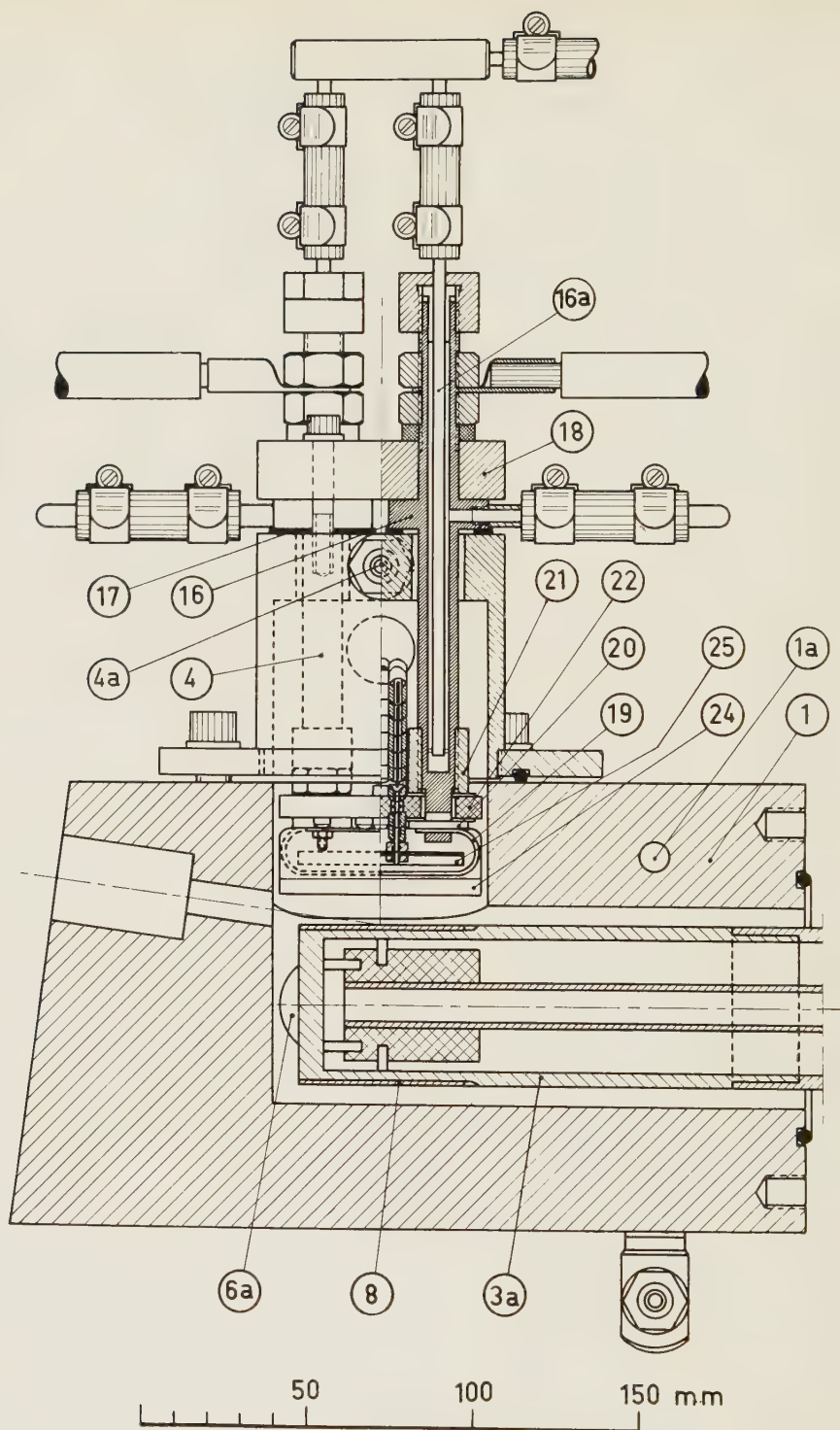


Fig. 22. Longitudinal section through the cathode and the central part of the roentgen tube.

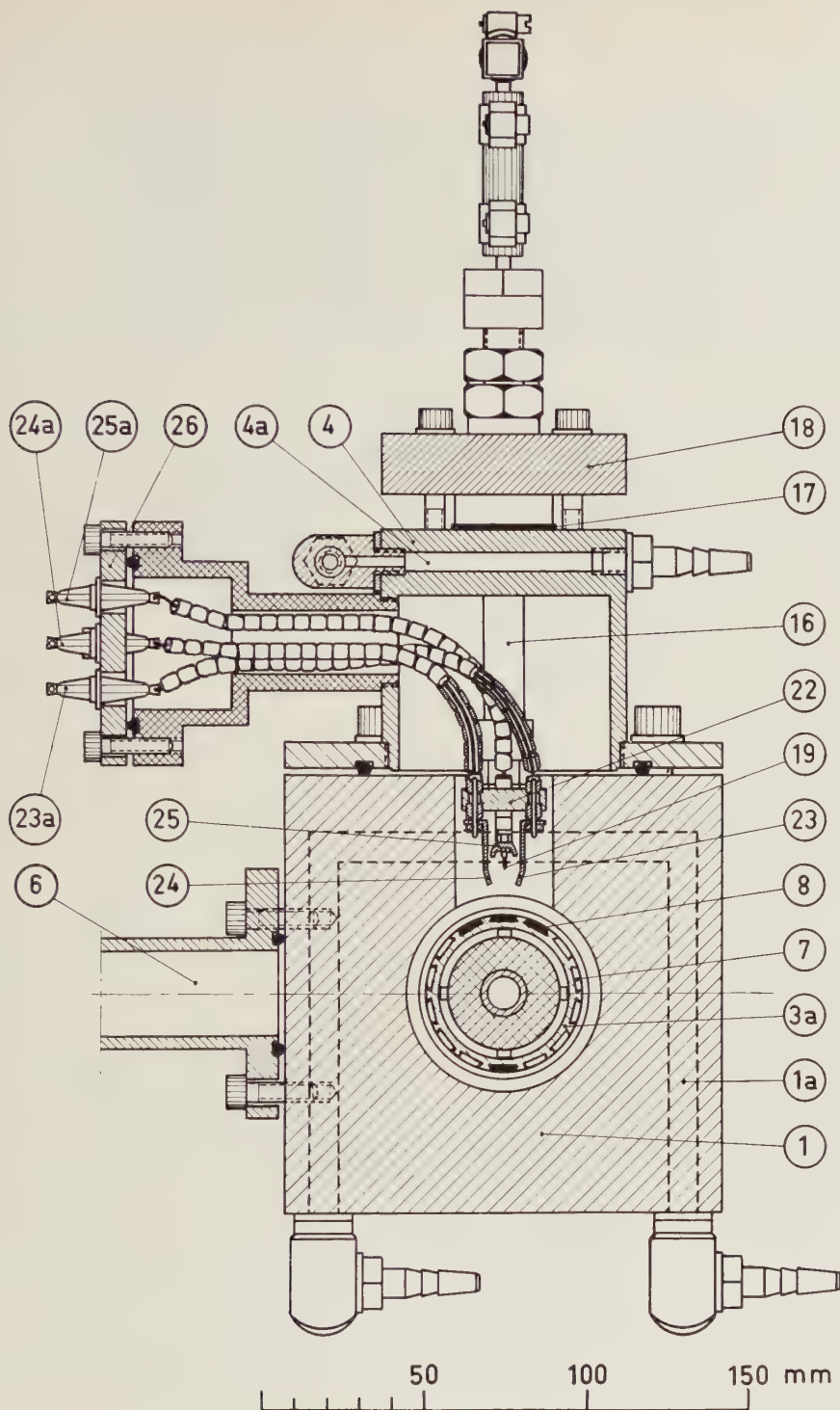


Fig. 23. Cross section through the roentgen tube.

or tungsten was used as target material in order to obtain an extremely soft roentgen spectrum with as high intensity as possible at a given voltage and a constant electron current in the roentgen tube. (3 *a*) is soldered to the outer part (3 *b*) of the anode, a brass tube, which is guided by the brass cylinder (9). By means of the O-ring (10 *a*), the metal ring (10) with a collar for the O-ring, and the threaded lock ring (11), the anode can be rotated and also be adjusted in the axial direction. The position of the different anode materials are indicated on the threaded metal ring (12), which is locked by (13). The two clamps (14) serve as an additional locking of the anode, which is water-cooled with the water flow through the axial tube (15).

The central part (4) of the *cathode* is water-cooled (4 *a*). The two inlets (16) for the filament current are insulated from (4) by means of the rubber gaskets (17) and the ebonite plate (18). The inlets are water-cooled with the water flow through (16 *a*). The filament (19) consists of a thoriated tungsten wire with a diameter of 1.0 mm, which is passed through cut holes in the inlets (16). It is clamped by two small cotters (20), which are pressed against the filament by means of the nuts (21) and the metal plate (22). The straight portion of the filament has a length of 50 mm. The filament is very durable and can generally be used for several hundred hours.

By means of thin mica sheets one inlet (16) is insulated from the plate (22), which is grounded through the other inlet (16). Three *focusing sheets*, (23), (24), and (25), made of nickel plate with a thickness of 0.5 mm, are attached to the metal plate (22) by means of insulating steatite pearls. Such pearls are also utilized to insulate the copper wires connecting the focusing sheets with the glass insulated inlets (Philips 88014—15), (23 *a*), (24 *a*), (25 *a*), which are soldered to the metal plate (26). Pin-hole photographs of the focal spot are shown in Fig. 24 a—g. The pictures show that, with the focusing sheets grounded, the size of the focal spot is constant, when the voltage across the roentgen tube does not exceed about 3.5 kV. At 5.0 kV across the roentgen tube, however, the sheets (23) and (24) must have a potential of about -150 volts to prevent an increase of the size of the focal spot which is about 1×6.5 mm.

The space charge of electrons in the roentgen tube is influenced by the shape of the focusing sheets and determines the maximum electron current in the roentgen tube at different voltages. At a voltage of 0.6, 1.0, and 1.5 kV, respectively, the corresponding maximum currents are about 10, 20, and 45 mA.

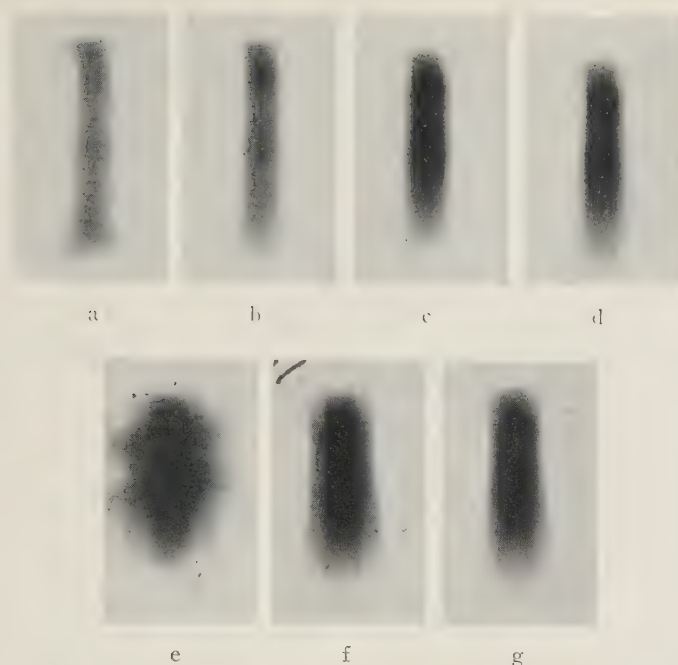


Fig. 24 a—g. Pin-hole photographs, $\times 4.3$, of the focal spot at different voltages across the roentgen tube and with different potentials applied to the focusing sheets (23) and (24). The sheet (25) grounded.

- a) Voltage across the roentgen tube: 1.5 kV. (23) and (24) grounded.
- b) 2.5 kV. (23) and (24) grounded.
- c) 3.5 kV. (23) and (24) grounded.
- d) 3.5 kV. — 50 volts applied to (23) and (24).
- e) 5.0 kV. (23) and (24) grounded.
- f) 5.0 kV. — 100 volts applied to (23) and (24).
- g) 5.0 kV. — 150 volts applied to (23) and (24).

A cross section through the *specimen holder* is shown in Fig. 25. The metal disc (27) with the biologic object and the reference system is pressed against the fine-grained photographic emulsion (28), when (29) is screwed on to (30). The tap (31) prevents the rotation of (30). The metal casing (32) protects the film against visible radiation, and a thin layer of aluminium (33) covers the opening through which the roentgen radiation passes. The window (33) has been composed of two thin aluminium foils with a total weight of $0.33 \text{ mg} \cdot \text{cm}^{-2}$. When the voltage across the roentgen tube is lower than 1.5 kV, the aluminium foil only serves to protect the photographic film against visible radiation from the hot filament cathode. Therefore, as thin

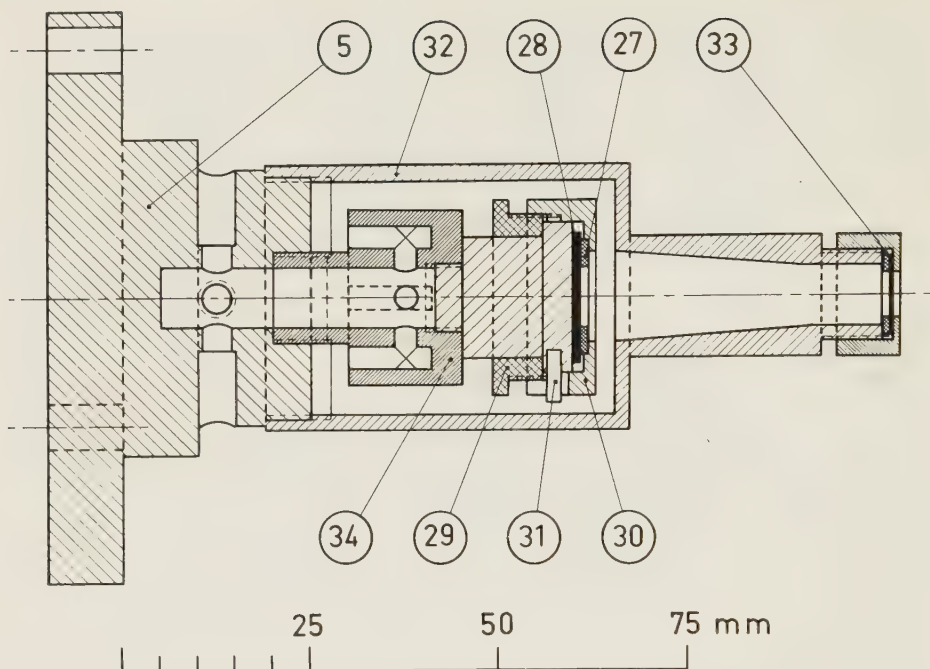


Fig. 25. Longitudinal section through the specimen holder.

a window as possible should be utilized in order to minimize the absorption of the soft roentgen radiation emitted in the roentgen tube. The thin aluminium foils break rather often, but as they are mounted on the specimen holder, small holes are easily discovered. The two metal parts (5) and (34) form a light trap through which the air in the specimen holder is evacuated. By means of additional brass tubes (35) in Fig. 21 the distance between the specimen and the focal spot of the roentgen tube can be varied within wide limits. In most of the experiments the distance between the focal spot and the specimen was 185 mm.

B. The Vacuum System and the High Voltage Supply

The vacuum system consists of a Siegbahn molecular pump (LKB 3009—G) backed by a two stage rotary mechanical fore-pump (Pfeiffer No. 30100). The vacuum is measured by a Pirani vacuum gauge.

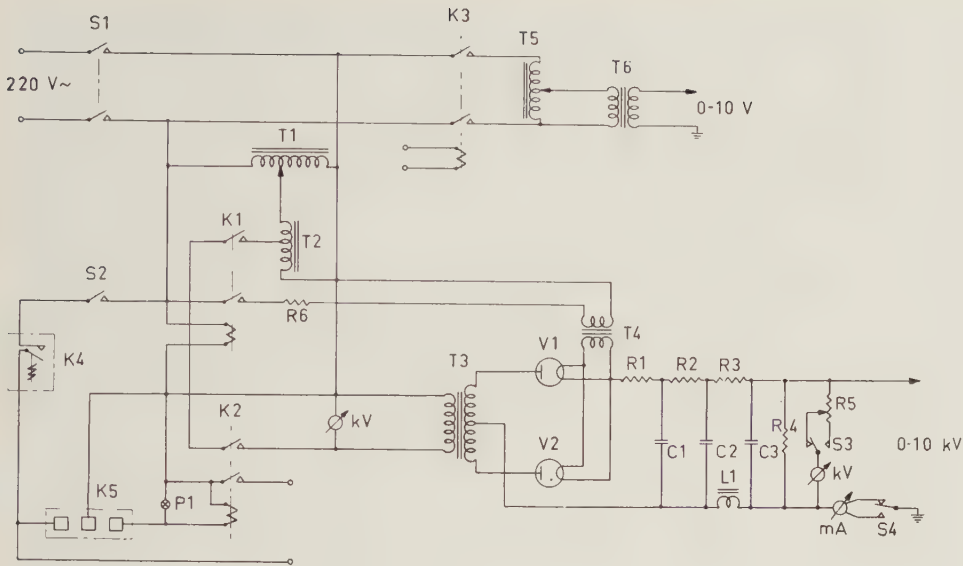


Fig. 26. Wiring diagram of the high voltage supply.

$R1 = R2 = R3 = 5 \text{ k}\Omega$

$R4 = 10 \text{ M}\Omega$

$R5 = 200 \text{ M}\Omega$

$R6 = 20 \Omega$

$C1 = C2 = 0.3 \mu\text{F}$

$C3 = 4 \mu\text{F}$

$L1 = \text{filter choke} \approx 0.1 \text{ H}$

$V1 = V2 = 200 \text{ Y (Schönander) rectifier tube}$

$T1 = \text{adjustable transformer } 220/0-250 \text{ volts}$

$T2 = \text{auto transformer } 0-70 \text{ volts}$

$T3 = \text{high tension transformer } 1/2 \times 150 \text{ volts}$

$T4 = \text{filament transformer for the rectifier tubes } 30:1$

$T5 = \text{adjustable transformer } 220/0-250 \text{ volts}$

$T6 = \text{filament transformer for the roentgen tube } 0-250/0-10 \text{ volts}$

$K1 = \text{contactor for the filament current of the rectifier tubes}$

$K2 = \text{contactor for the high tension transformer}$

$K3 = \text{contactor for the filament current of the roentgen tube}$

$K4 = \text{cooling water relay}$

$K5 = \text{time relay}$

$P1 = \text{pilot light for high tension}$

$S1 = \text{switch for } K1$

$S2 = \text{switch for } K2$

$S3 = \text{reversing switch for the kV-meter } 0-5/0-10 \text{ kV}$

$S4 = \text{reversing switch for the mA-meter } 0-30/0-300 \text{ mA}$

The wiring diagram of the high voltage supply is shown in Fig. 26. Full-wave rectification is utilized and great capacitances are connected parallel with the roentgen tube to smooth the voltage across the tube. No stabilizing circuit was utilized, as the variations of the electron current in the roentgen tube are small, and the exposure times are generally short. A relay to control

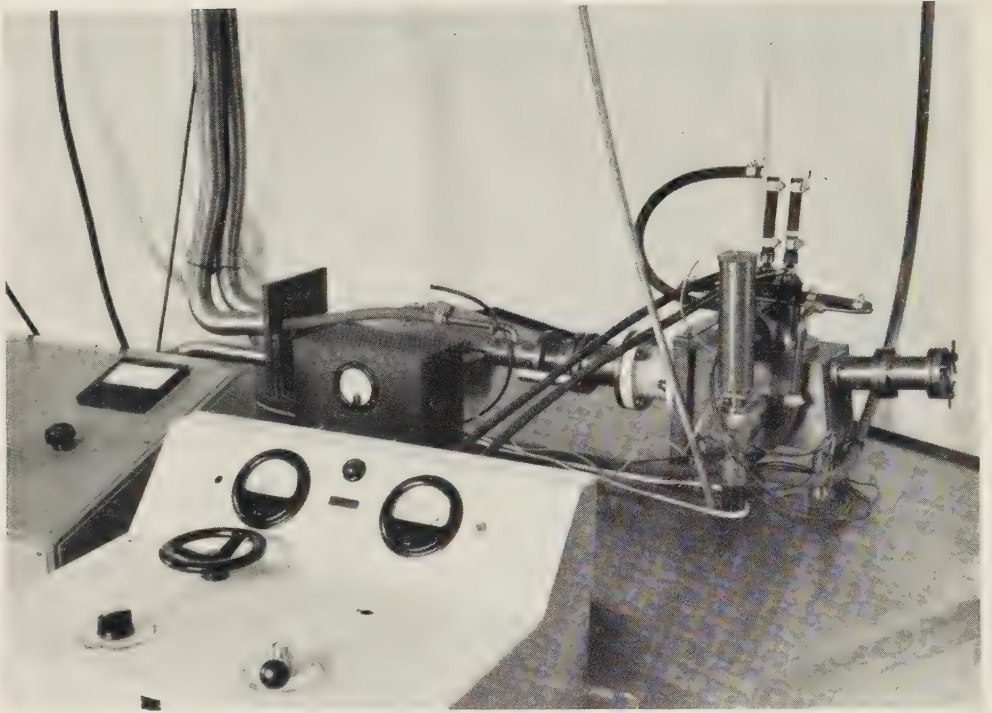


Fig. 27. View of the roentgen tube and high voltage supply for total dry weight determinations.

the flow of cooling water is connected in the circuit of the primary side of the high voltage transformer.

The anode has a casing of insulating bakelite plates, and the rubber tubes for the cooling water of the anode are enclosed in grounded Plica tubes. A photograph of the roentgen tube and the high voltage unit, utilized for total dry weight determinations of small biologic objects, is shown in Fig. 27.

CHAPTER 8

The Experimental Procedure

In the first section the statistical methods utilized to evaluate the experimental, accidental errors are presented. The technique of quantitative micro-radiographic determination of the total dry weight of small biologic objects will be described in the following sections. In the last section the combined influence of the different errors on the experimentally determined weight of a biologic structure per unit area is discussed.

A. Statistical Methods

The accidental variations of experimentally determined quantities in the present work have an approximately *normal* distribution around the arithmetic mean in question. Therefore, the experimental errors have been calculated according to the usual formula for the *standard deviation* of a quantity and according to the Gauss law of propagation of standard errors.

$$\sigma_x = \pm \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} \quad \dots\dots\dots (65).$$

σ_x = standard deviation of x .

$x - \bar{x}$ = deviation of the individual determination of x from the arithmetic mean $\bar{x}$.

n = number of determinations of x .

If

$$X = f(x_1, x_2, x_3, \dots, x_n) \quad \dots\dots\dots (66),$$

the *Gauss law of propagation of standard errors* [158, pp. 23—26] has the following form:

$$\varepsilon_X^2 = \sum_{k=1}^n \left(\frac{\partial X}{\partial x_k} \varepsilon_{x_k} \right)^2 \quad \dots\dots\dots (67).$$

ε_X = standard error of X .

ε_{x_k} = standard error of x_k .

When equation (67) is applied to the standard error $\varepsilon_{\bar{x}}$ of the *arithmetic mean* $\bar{x}$ of n determinations of the quantity x , $\frac{\partial X}{\partial x_k} = \frac{1}{n}$ and $\varepsilon_{x_k} = \sigma_x$. Hence

$$\varepsilon_{\bar{x}} = \frac{\sigma_x}{\sqrt{n}} \dots\dots\dots (68).$$

For the standard error ε_d of the *difference between duplicates* $\frac{\partial X}{\partial x_k} = 1$, $\varepsilon_{x_k} = \sigma_x$, and $n=2$. If no correlation exists between the first and the second determination, and if the mean difference $\bar{d}$ does not differ significantly from naught, the following expression is obtained according to equation (67) :

$$\varepsilon_d = \sigma_x \sqrt{2} \dots\dots\dots (69).$$

For a product with the general factor $(x_k)^{z_k}$, the partial derivative is given by

$$\frac{\partial X}{\partial x_k} = z_k (x_k)^{z_k-1} \frac{X}{(x_k)^{z_k}} = \frac{z_k X}{x_k} \dots\dots\dots (70).$$

Equation (67) then takes the following form

$$\varepsilon_X^2 = \sum_{k=1}^n \left(\frac{z_k X \varepsilon_{x_k}}{x_k} \right)^2 \dots\dots\dots (71),$$

which can be transformed to

$$\left(\frac{\varepsilon_X}{X} \right)^2 = \sum_{k=1}^n \left(z_k \frac{\varepsilon_{x_k}}{x_k} \right)^2 \dots\dots\dots (72).$$

$100 \varepsilon_X/X$ and $100 \varepsilon_{x_k}/x_k$ are the *coefficients of variation* of X and x_k , respectively, i.e. the relative standard errors, expressed in per cent.

B. The Preparation of the Specimen

The specimen, generally a microtome section of biologic material, is mounted on a metal disc with a slit, $0.5\text{--}0.6 \times 8$ mm, covered with a zapon varnish foil with a weight of about $0.2 \text{ mg} \cdot \text{cm}^{-2}$ (see Fig. 16). The supporting

foil is made by spreading a few drops of diluted zapon varnish solution on a clean surface of distilled water in a small glass dish, on the bottom of which the metal discs have been placed on a perforated metal plate. When the zapon varnish foil on the water surface begins to wrinkle, the discs on the metal plate are raised out of the water, and the foil is allowed to dry on the discs. Foils with interference fringes are discarded.

In the present work most of the biologic material has been fixed in 10 per cent neutral formalin or frozen-dried according to the technique described by MOBERGER, LINDSTRÖM and ANDERSSON [172]. The microtome section is stretched on a small drop of cold, distilled water on one half of the slit. No measurable decrease of the dry weight of biologic structures in frozen-dried, paraffin-embedded sections was obtained after rapid stretching of the paraffin sections on a water surface. The water is sucked away, and the specimen is dried for at least 6 hours at 37° C. Otherwise the section easily slips off the holder during the deparaffinizing, performed in chloroform for 15 to 20 minutes.

Freeze-drying has also been performed on biologic objects in the form of smear preparations, made on thin Parlodion foils on cover slips. Pieces of the Parlodion foil with the frozen-dried specimen have then been transferred to the supporting foil on the metal disc, thus avoiding the embedding procedure and deparaffinizing.

C. The Procedure of Making the Reference System and Its Accuracy

The reference system, consisting of a step wedge of nitrocellulose foils, can be made according to different methods. In the original procedure [30] a few drops of a zapon varnish solution was spread on a water surface as described in the previous section, and the foil was lifted by means of a wire loop. The weight per unit area was determined by a gravimetric method with a coefficient of variation of ± 11 per cent [163]. More elaborate methods have been used to prepare even foils on water [118, 151], and interferometric methods have been utilized to determine the thickness of the foils [152, 136]. CLEMMONS *et al.* [128] use Parlodion foils made on glass surfaces, and from a known admixture of a dye the thickness of the foils is determined by colorimetric analysis.

The nitrocellulose foils, utilized by the author, are made on glass, but the weight per unit area of one foil is determined by a gravimetric method. A

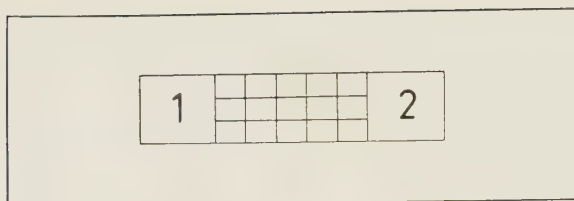


Fig. 28. The cutting of the reference system.

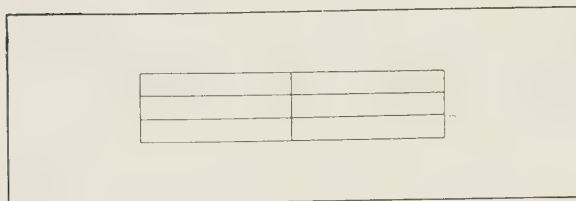


Fig. 29. The cutting of the reference system when testing the evenness.

carefully cleaned microscopic slide of highest quality is dipped in a solution of 2.5 per cent Parlodion in amyl acetate. The slide is allowed to dry in the vertical position with a long edge resting against a filtering paper. An even number of dippings is made, and the slide is dried for half an hour after each dipping, resting on the same long edge every other time. Generally four dippings are made, giving a foil with a weight of about $0.15 \text{ mg} \cdot \text{cm}^{-2}$, which is appropriate when analysing biologic specimens with a thickness of between 5 and 10μ .

The dried Parlodion foil on the slide is cut as shown in Fig. 28. The slide is placed on a microscope stage provided with vernier readings. The edge of a small knife, mounted on a special holder at the position of the microscope objective, can be adjusted parallel with the directions of the movements of the microscope stage. The foil on the glass can then be accurately cut by moving the microscope stage.

When the slide is immersed in water, the cut pieces of the Parlodion foil are floated free. The large pieces, marked 1 and 2, with an area of 0.90 cm^2 , are dried and weighed on a torsion balance. The 15 small pieces, each with an area of 0.12 cm^2 , are used to build the step wedge on the supporting foil on the metal disc. A small drop of water is placed on the supporting foil over the slit close to the deparaffinized specimen, and one of the small pieces of the Parlodion foil is transferred to this drop. The reference foil is then

allowed to dry on the disc, whereupon the procedure is repeated until an appropriate number of steps (generally four) in the wedge has been obtained.

When utilizing a gravimetric procedure the weight per unit area of one foil, i.e. w in equation (62), is immediately obtained. The foils must have an even thickness, however, as areas adjacent to those weighed constitute the wedge. The weights of the areas, marked 1 and 2 in Fig. 28, in different reference systems constitute a series of duplicates. The coefficient of variation of the difference between duplicates was ± 3.21 per cent in a series of 36 foils, used as reference systems. According to equation (69) the coefficient of variation for a single determination is then ± 2.27 per cent, and for the mean of two determinations of the weight per unit area of the foil ± 1.6 per cent is obtained according to equation (68).

As a further control of the evenness of the Parlodian foils made on microscopic slides, foils were cut as shown in Fig. 29, and the small pieces, each with an area of 0.60 cm^2 , were weighed. The coefficient of variation of the weight per 0.60 cm^2 was determined for 10 different foils, and the arithmetic mean of the coefficients was ± 1.9 per cent.

Three factors influence upon the coefficient of variation of the weight per unit area of the foil, *viz.* the errors of the cutting and weighing procedures, and small unevennesses in the foils. The weighed areas of the foils are comparatively large, and small local variations within the foils may be smoothed out. However, the coefficients of variation of the photo-currents, corresponding to the absorption image of the reference system in the microradiogram, can be utilized as a measure of the evenness of the foils within small areas, which is discussed in the following section.

D. The Photometric Procedure

The roentgen absorption image of the specimen and the reference system is recorded on a fine-grained photographic emulsion in close contact with the object (see Fig. 16). The light transmissions in the developed microradiogram are determined by means of direct photometry of the optically enlarged image of the microradiogram.

In the present work the magnifying system was a Leitz Ortholux microscope with apochromate objectives (4 mm, numerical aperture 0.95, or 2 mm, immersion, numerical aperture 1.32) and periplane oculars (6x or 8x). The light source was a sound film tungsten filament lamp, connected to an

accumulator by soldered wires and with a step potentiometer as a variable series resistance. The output voltage of the accumulator was kept constant by means of a load-levelling relay, connected to the line AC. The enlarged image of the microradiogram was focused on a screen immediately in front of the housing of the photomultiplier tube (RCA 931-A). A pin-hole with a diameter of 2 mm made it possible to project different selected areas in the microradiogram on the photoelectric surface of the photomultiplier tube. The power supply for the photomultiplier tube was of the type, described by ENGSTRÖM and WEGSTEDT [146], and a Multiflex galvanometer recorded the anode current, which is proportional to the intensity of the incident light.

The anode current was kept below 1 μA in order to avoid fatigue of the photomultiplier tube. The linearity of the photometric response to the intensity of incident light was tested by photometry of plates with known densities. The errors of the photometer are discussed in connection with the other factors influencing upon the coefficient of variation of the photo-current.

In an investigation of the distribution of the total dry weight in squamous epithelium during carcinogenesis [166, 167, 170] 95 microradiograms of different specimens were identically exposed to roentgen radiation and registered on Kodak Maximum Resolution plates. The microradiograms were mounted on cover slips with Permunt as embedding medium between the photographic emulsion and the cover slip. As the glass in the photographic plates has the same thickness as ordinary microscopic slides, the mounted microradiograms fulfil the requirements for optimum optical conditions.

The photometric procedure was constant in the whole series of microradiograms, the area of the aperture in front of the photomultiplier tube corresponding to an area of 7 μ^2 in the original microradiogram. The photo-current was determined in 10 points of measuring in each of the areas with densities, corresponding to the intensity of the incident roentgen radiation and to the intensity of roentgen radiation transmitted through one, two, and three reference foils, respectively. The standard deviation of the photo-current for each area was calculated. For these four areas the arithmetic means of the photo-currents and of the corresponding standard deviations were calculated in the series of microradiograms, and the values are presented in Table XIII.

The increase of the absolute mean value of the standard deviation with increasing photo-current is small, indicating that the foils in the reference system are very even within small areas. However, several factors influence

Table XIII. Mean values of the standard deviation of the photo-current, corresponding to different areas in the microradiograms

Area in the microradiograms with a density corresponding to	intensity of incident roentgen radiation	intensity of roentgen radiation transmitted through		
		1 foil	2 foils	3 foils
Mean value of the photo-current (μA) n = 95	~ 0.47	~ 0.61	~ 0.78	~ 0.95
Mean value of the standard deviation of the photo-current (μA) n = 95	$\pm (0.0072 \pm 0.0002)$	$\pm (0.0085 \pm 0.0002)$	$\pm (0.0098 \pm 0.0002)$	$\pm (0.0107 \pm 0.0002)$

upon the standard deviation of the photo-currents corresponding to the theoretically constant densities of these four different areas.

1) *Inhomogeneous distribution of the intensity in the incident roentgen beam.* High intensities of extremely soft roentgen radiation can be generated by the constructed roentgen tube. The distance between the focal spot and the specimen can therefore be large, despite the utilization of extremely insensitive, fine-grained photographic emulsions to register the roentgen absorption images. At a distance of 185 mm the time of exposure has been between 6 and 10 minutes at a voltage of 1.5 kV and an electron current in the roentgen tube of 45 mA. A homogeneous intensity of roentgen radiation has then been obtained over the whole metal disc, supporting the object.

2) *Variations of the thickness of the supporting zapon varnish foil.* Within the small areas corresponding to the slit in the metal disc the supporting foils have been uniform in thickness, but the exact value of the contributing error owing to this factor has not been determined separately.

3) *Variations and artefacts in the fine-grained photographic emulsions.* The fine-grained photographic emulsions tested have shown great differences in this respect. Thus the Lippmann emulsions have been far inferior to Kodak Maximum Resolution and Kodak High Resolution emulsions. These latter emulsions are extremely homogeneous over large areas with a minimum of artefacts. Furthermore, as the Lippmann emulsions are manufactured on a celluloid base, the optical conditions for these films at the photometric pro-

cedure are not as good as for plates mounted on cover slips. However, the plates must be cut in small squares (12×12 mm) with the utmost care to avoid small glass particles on the photographic emulsion.

4) *The granularity of the photographic emulsions.* Owing to the statistical spatial distribution of the developed grains in a photographic emulsion, the number of grains (n) per unit area has an absolute error of $\pm \sqrt{n}$. The corresponding relative error, $\pm 100/\sqrt{n}$ per cent, is of importance for the photographic-photometric procedure. When determining the photo-current corresponding to a *particular* area in the roentgen absorption image, the error, introduced by the granularity of the photographic emulsion, can be minimized by utilizing fine-grained photographic emulsions of highest quality and the most suitable developing conditions. These factors are discussed in more detail in section E. For a given photographic emulsion and a standardized procedure of development the smallest area to be measured is determined by the allowed relative error, $\pm 100/\sqrt{n}$ per cent. Utilizing Kodak High Resolution emulsions the measured area was only $1.5 \mu^2$ in some biologic applications. The photo-currents, corresponding to the intensity of incident roentgen radiation and to the intensity of radiation transmitted through the reference system, had a lower standard deviation than those presented in Table XIII, which were obtained for Kodak Maximum Resolution emulsions and a measured area of $7 \mu^2$.

5) *Errors of the photometer.* Small variations of the intensity of the light source occur during an initial period of 30 minutes, but after this space of time the variation is very small. The voltage from the power supply was well stabilized [146], and the error of the response of the photomultiplier tube was negligible. However, no experiments were performed to obtain a separate value of the errors of the photometer, which are included in the standard deviations of the photo-currents in Table XIII.

6) *Variations of the weight per unit area of the reference foils.* Owing to small inhomogeneities of the reference foils, an additional error is included in the standard deviations of the photo-currents corresponding to the image of the reference system in the microradiograms. This can be seen in Table XIII, which has been discussed previously.

The combined effect of these different factors determine the standard deviations of the photo-currents in Table XIII. The different errors have been reduced as far as possible in order to increase the accuracy and the resolution of the method.

E. The Spatial Resolving Power of the Microradiographic Technique

The spatial resolving power of the microradiographic technique is given by the distance between two structures in the object which are barely separated in the microradiogram. When the roentgen absorption image of an object is registered in the scale 1:1 and secondarily magnified by photo-micrography, the *geometric unsharpness* in the microradiogram and the *graininess* of the photographic emulsion determine the spatial resolving power, provided the secondarily magnifying optical system is of high quality, i.e. apochromate immersion objectives and periplane oculars.

The *geometric unsharpness* P is schematically illustrated in Fig. 30, where f is the longest diameter of the focal spot, s the distance between the focal spot and the specimen, r_1 the thickness of the specimen, and r_2 the thickness of the photographic emulsion. The following equation, relating these quantities, is obtained:

$$P = \frac{f(r_1 + r_2)}{s} \dots\dots\dots (73).$$

In the roentgen tube, described in Chapter 7, f has a value of 6.5 mm and s has been 185 mm in most of the experiments. The thickness of the specimen has generally been 5 μ . The value of r_2 is only a few microns, when utilizing the extremely fine-grained Kodak High Resolution and Kodak Maximum Resolution emulsions. Furthermore, the extremely soft roentgen radiation is mainly absorbed in the surface layer of the photographic emulsion. If r_2 is neglected, P has the value 0.18 μ , and if r_2 is assumed to be about 3 μ , the corresponding value of P is 0.28 μ .

In the utilized procedure the geometric unsharpness is of the same magnitude as the resolving power of the ordinary light microscope, and hence the *graininess* of the fine-grained photographic emulsions is of decisive

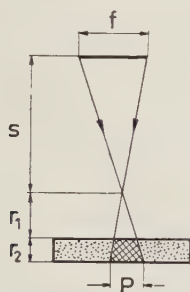


Fig. 30. The geometric unsharpness of the microradiographic technique.

importance for the spatial resolving power of the microradiographic technique. The graininess is the term for the *subjective* impression of nonuniformity of a magnified image of a homogeneously exposed area of the photographic emulsion [154]. This factor was discussed in an earlier investigation of the properties of fine-grained photographic emulsions used for microradiography [143]. However, the *objective* effect of the nonuniformity of the utilized photographic emulsion on the quantitative microradiographic analyses is more important. In this case the term *granularity* of a photographic emulsion is used [154]. The graininess and the granularity are related to each other, though different methods are utilized to evaluate these two properties of a photographic emulsion.

In the present work the standard deviation of the photo-current, corresponding to a homogeneously exposed area in the microradiogram, has been utilized as a measure of the granularity of different fine-grained photographic emulsions and of the same photographic emulsion for different developing conditions. With such a procedure variations of the thickness of the photographic emulsion will also contribute to the standard deviation of the photo-current, but for quantitative purposes this factor is as important as the pure effect of granularity. All other factors influencing upon the standard deviation of the photo-current, discussed in the previous section, were kept as constant as possible. A 2 mm apochromate immersion objective with a numerical aperture of 1.32 was used at the photometry, each measured area with a size of $1.5 \mu^2$ in the original microradiogram.

In a preliminary investigation a number of different developers were tested on the three most fine-grained photographic emulsions available, Kodak High Resolution plates, Kodak Maximum Resolution plates, and Lippmann films. The three most suitable developers could be selected from visual comparison of the graininess in high power photo-micrographs of differently developed microradiograms.

A step wedge consisting of six Parlodion foils was then utilized as a test object to obtain a series of different densities in the same microradiogram. The exposures to roentgen radiation, generated at 1.5 kV and filtered through aluminium ($0.33 \text{ mg} \cdot \text{cm}^{-2}$), were adjusted to a maximum density of about 0.45 in one series of microradiograms, and to a maximum density of about 1.0 in a second series. On each homogeneously exposed area 20 photometric measurements were performed, and the standard deviation of the photo-current was calculated. At densities lower than 0.5 the values of the different

Table XIV. Standard deviations of photo-currents for fine-grained photographic emulsions and different developers

Photographic emulsion	Development (18° C)		Mean value of the standard deviation of the photo-current (μA) n = 11
	Kodak Developer	Time	
Kodak High Resolution plates (CDC1D4; 50 31)	D-76	20'	$\pm (0.0159 \pm 0.0008)$
—, —	D-19	6'	$\pm (0.0147 \pm 0.0008)$
—, —	D-158 (diluted 1 : 1)	6'	$\pm (0.0109 \pm 0.0004)$
Kodak Maximum Resolution plates (S.P.C. 2802E)	—, —	6'	$\pm (0.0110 \pm 0.0004)$
Lippmann film (31005)	—, —	6'	$\pm (0.0216 \pm 0.0013)$

standard deviations, corresponding to a certain emulsion developed in a particular way, were approximately randomly spaced and independent of the density of the area. The absolute mean values of the photo-currents for these areas varied between 0.35 and 0.90 μA . At densities higher than 0.5, however, the standard deviation of the photo-current decreased continuously at increasing densities. For areas with densities lower than 0.5, mean values of the standard deviations of the photo-currents for the different photographic emulsions and for the three most suitable developers are presented in Table XIV. Each mean value has been calculated from the standard deviations corresponding to 11 areas with a total of 220 photometric measurements. The standard errors of the different means are also included.

Thus the granularity of Kodak High Resolution plates is lowest when the developer Kodak D-158 is used. With respect to the granularity some Kodak Maximum Resolution emulsions are equivalent to Kodak High Resolution emulsions, though these latter emulsions generally have a lower granularity. The Lippmann emulsions tested have been far inferior to the two Kodak emulsions. Kodak D-158 has the following composition:

Hot water (50° C)	750 ml
Elon	3.2 g
Sodium sulphite, cryst.	100 g

Hydroquinone	13.3 g
Sodium carbonate, cryst.	186 g
Potassium bromide	0.9 g
Water to make	1000 ml

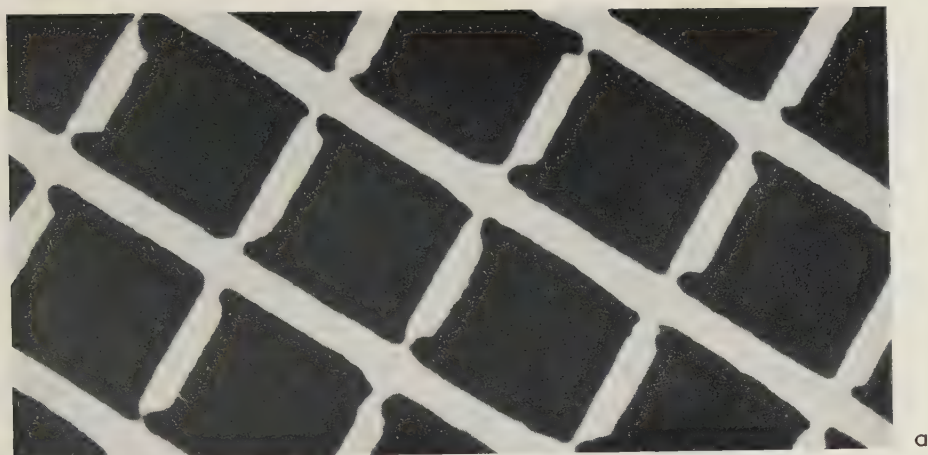
One part stock solution is diluted with one part water. Development is performed for 6 minutes at 18° C.

The values in Table XIV are no absolute measures of the granularity of the different photographic emulsions, as all the factors discussed in the previous section contribute to the standard deviation of the photo-current. As these factors were kept constant, however, *relative, objective measures of the granularity* of these extremely fine-grained photographic emulsions were obtained. The experimental procedures necessary to obtain the optimum spatial resolving power have been utilized in all applications. Some biologic applications, illustrating the absolute values of the spatial resolving power of the quantitative microradiographic technique, are presented in Chapter 9.

F. The Quantitative Resolving Power of the Microradiographic Technique

The quantitative resolving power of the microradiographic technique is defined as the smallest amount of organic material per unit area that can be determined, or the smallest detectable difference between the total dry weights of different structures. If the photometric procedure is constant, the contrast in the roentgen absorption image, registered on the fine-grained emulsion, is the most important factor influencing upon the quantitative resolving power.

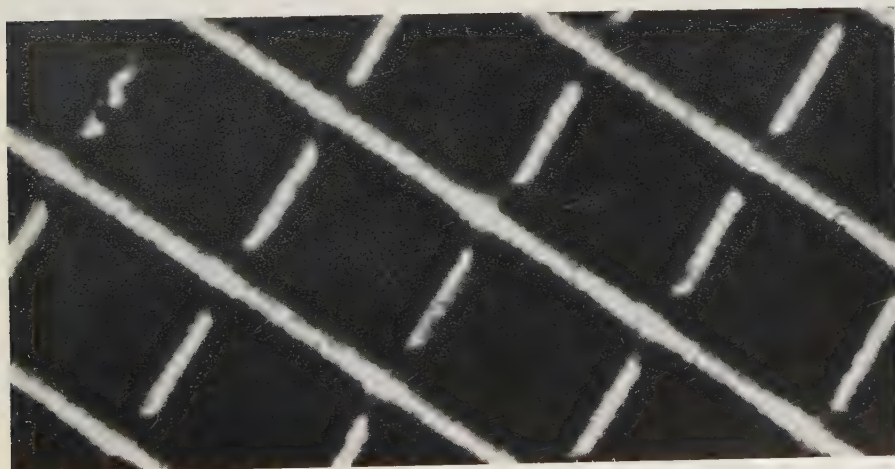
The contrast obtained in the microradiogram is determined by the gamma value of the photographic emulsion and the differences between the intensities of roentgen radiation transmitted through cytologic structures with different amounts of total dry weight per unit area. The fine-grain development necessary to obtain an optimum spatial resolving power gives a relatively low gamma value. The other factor influencing upon the contrast, however, varies with the voltage across the roentgen tube. As the mass absorption coefficient is approximately proportional to the third power of the wavelength of the roentgen radiation, with the exception of the absorption edges, the composite mass absorption coefficient of biologic material increases, when the voltage across the roentgen tube is decreased, which has been discussed in the introduction in Chapter 6.



a

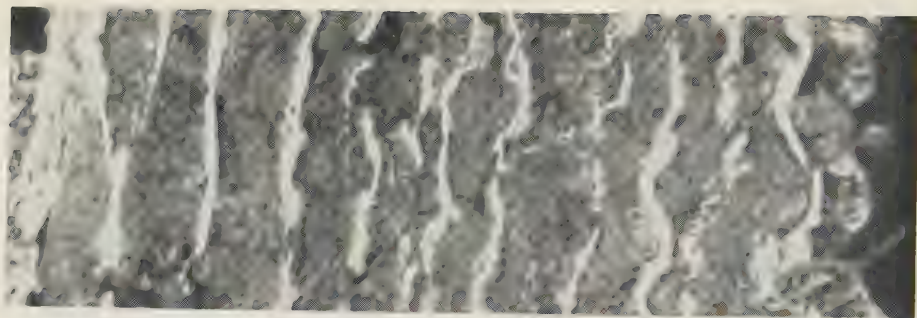


b

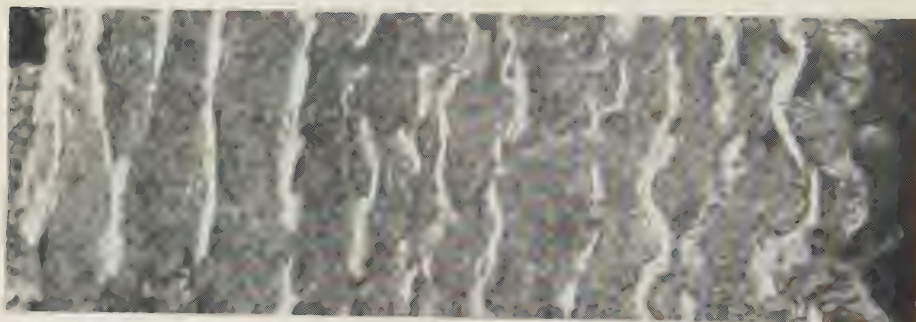


c

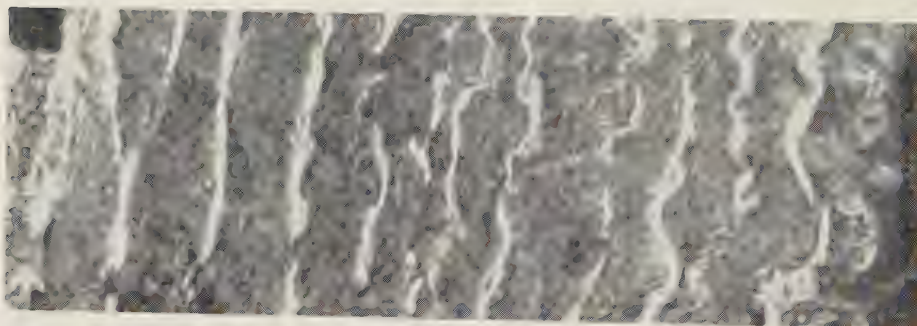
Fig. 31. Microradiogram, $\times 1500$, of a silver grid registered at
a) 1.5 kV, b) 5.0 kV, c) 8.0 kV.



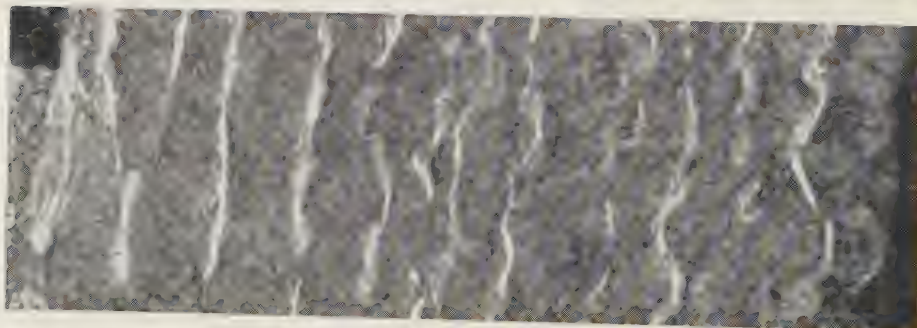
a



b



c



d

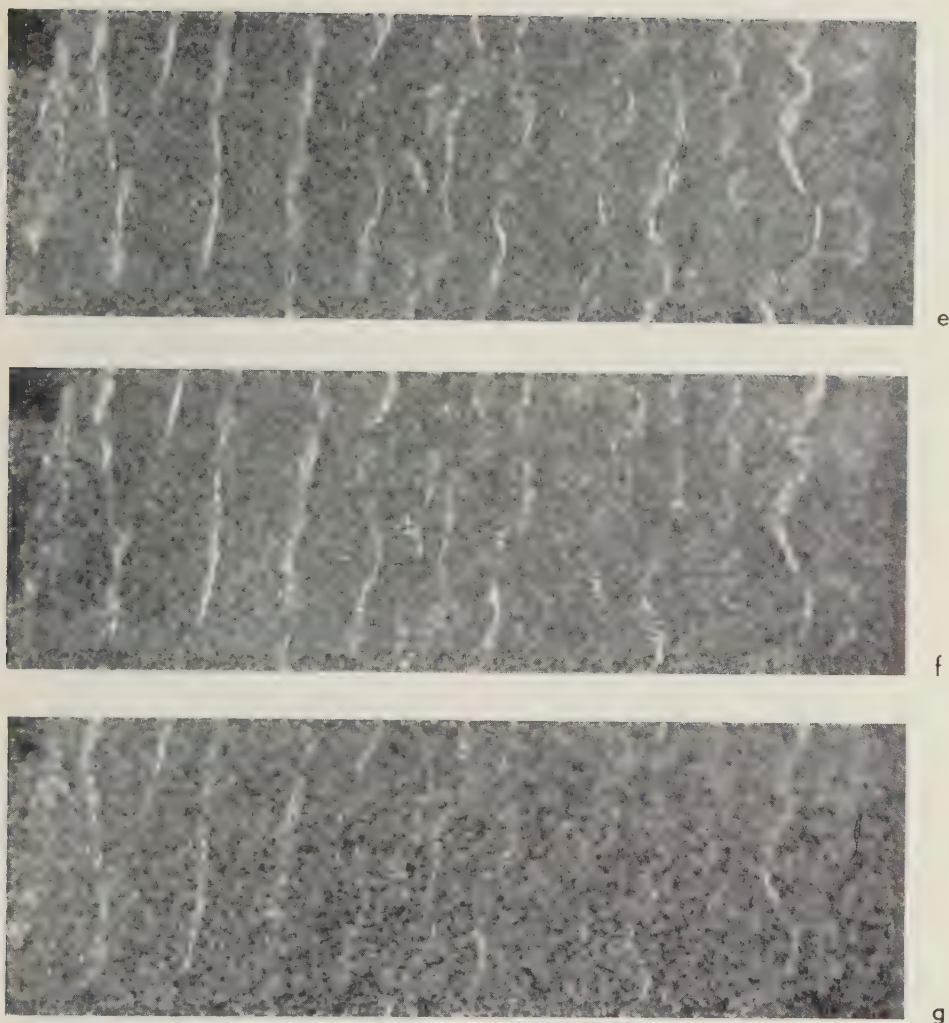


Fig. 32. Microradiogram, $\times 600$, of a $3\ \mu$ thick section of a frozen-dried carotid artery of the rabbit, registered at a) 0.8 kV, b) 1.0 kV, c) 1.2 kV, d) 1.5 kV, e) 2.5 kV, f) 3.5 kV, g) 5.0 kV.

A series of microradiograms of a silver grid, kindly supplied by Dr. V. E. COSSLETT, are shown in Fig. 31 a—c. The meshes have a diameter of $3.3\ \mu$, corresponding to 5.0 mm in the microradiograms, *secondarily magnified* 1 500 times. This diameter is exactly obtained in Fig. 31 a, where the microradiogram has been registered at 1.5 kV. The edges of the meshes which have a

relatively low weight per unit area, are not seen in the roentgen absorption image in Fig. 31 b, owing to the lower absorption of the continuous roentgen spectrum, generated at 5 kV. The meshes in the picture have a diameter of about 4 mm, and this decrease of the diameter is still more pronounced in Fig. 31 c, registered at 8 kV. This metal grid is a good test object for the geometric unsharpness of the experimental procedure, and *no adjacency effects* were found in the original microradiograms. However, the structures are too coarse to give information regarding the other factors influencing upon the spatial resolving power. No evaluation of the quantitative resolving power can be made utilizing such test objects.

The influence of the voltage across the roentgen tube upon the quantitative resolving power of the microradiographic technique is illustrated in Fig. 32 a—g and Fig. 33 a—f. *In all the photo-micrographs of microradiograms in this work the light areas correspond to structures with a high absorption of roentgen radiation, i.e. structures with a high total dry weight per unit area.* In the 3 μ thick section of the arterial wall the elastic lamellae, showing a high contrast when registered at low voltages, are barely visible in the microradiograms registered at 3.5 and 5.0 kV. The sharp absorption images of the red blood cells in the microradiograms registered at 0.8 and 1.0 kV contrast with the faint shadows of the cells obtained when the voltage across the tube exceeds about 2 kV.

When quantitative measurements are performed on microradiograms of the same object, registered at different voltages across the roentgen tube, the difference between the photo-currents, corresponding to two structures with a different amount of total dry weight per unit area, is greater in microradiograms registered at lower voltages. Consequently the accuracy and the quantitative resolving power of the microradiographic technique has a maximum when as soft a continuous roentgen spectrum as possible is utilized.

G. The Total Resolving Power and the Combined Experimental Error of the Quantitative Microradiographic Technique

The total resolving power of the microradiographic technique is given by the smallest determinable absolute weight of organic material. The smallest area to be analysed is determined by the spatial resolving power, and the quantitative resolving power determines the smallest measurable amount within that area. No absolute values of the total resolving power can be

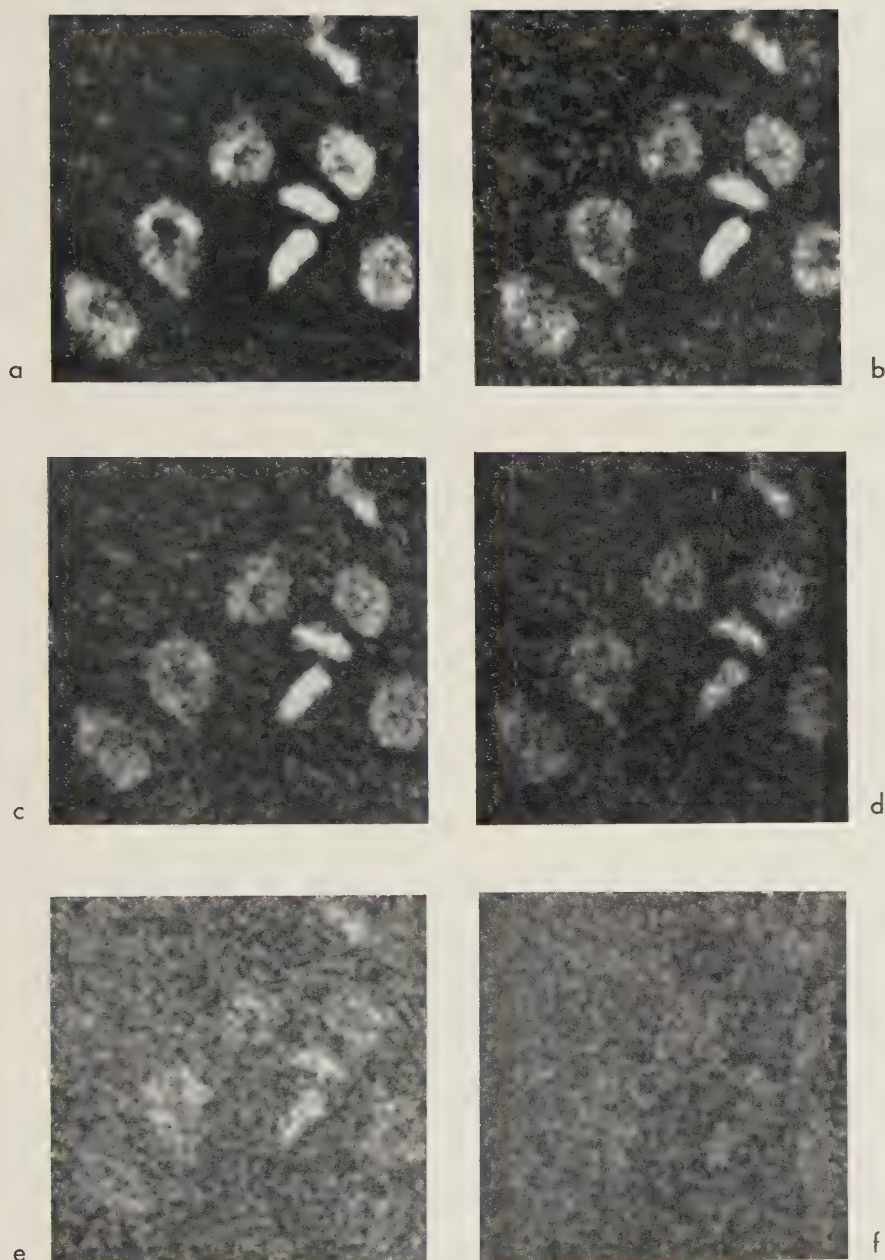


Fig. 33. Microradiogram, $\times 1500$, of frozen-dried red blood cells of the rabbit, registered at a) 0.8 kV, b) 1.0 kV, c) 1.2 kV, d) 1.5 kV, e) 2.5 kV, f) 5.0 kV.

given because of the varying influence of different biologic objects and the allowable error in each particular case. The total resolving power must therefore be determined experimentally for each biologic object investigated. Some quantitative measurements, presented in Chapter 9, have been performed on areas as small as $1.5 \mu^2$ in sections with a thickness of 3μ , corresponding to an analysed volume of about $5 \mu^3$.

If equations (62) and (64) are combined, and introducing the mean value 1.32 according to equations (64 b) and (64 c), the following equation is obtained:

$$(1 + \varepsilon) m_x = 1.32 u_x w \dots\dots\dots (74).$$

The magnitude of the *systematic error* ε , discussed in Chapter 6, varies for different biologic objects. With the exception of calcified tissues and biologic specimens with a high concentration of lipids, ε in equation (74) can be evaluated to about ± 0.03 . The error of the chemical analyses of the elementary composition of the reference system and the uncertainty of the values of the mass absorption coefficients contribute to the *error of the constant* 1.32. As the average animal protein and the reference system are composed of the same elements, and as only the ratio between their composite mass absorption coefficients are calculated, the errors of the mass absorption coefficients have only a small influence upon the error of the constant. Considering these different factors the total error of the constant 1.32 is about ± 0.01 .

The *coefficient of variation* of w has a value of about ± 1.6 per cent. The errors of the photo-currents corresponding to the reference system and the biologic structure, and the error when drawing the calibration curve, contribute to the *coefficient of variation* of u_x which must be determined from duplicates in each particular investigation.

In an investigation of the total dry weight of squamous epithelium during carcinogenesis [166, 167] the coefficient of variation of u_x was ± 4.1 per cent for a particular biologic structure. The combined experimental error of the weight per unit area was then $\pm \sqrt{4.1^2 + 1.6^2} = \pm 4.4$ per cent according to equation (72).

In order to obtain the weight per unit volume the thickness of the specimen must be determined. However, the methods of measuring the thickness of thin microtome sections are not accurate. When focusing a microscope on the upper and lower surfaces of a section with a thickness of about 5μ , LANGE

and ENGSTRÖM [162] obtained a coefficient of variation of ± 10 per cent in the experimental determination of the thickness. Therefore, in tissue sections, total dry weights of cytologic structures per unit area should be utilized as far as possible.

If the quantitative microradiographic procedure is combined with extraction and digestion experiments, the different fractions of organic material are obtained as differences between two determinations of the total dry weight of the biologic structure. The experimental error is then at least $\sqrt{2}$ times greater than the corresponding error of a single determination of the total dry weight.

Determinations of ratios between the weights per unit area of different structures in the same section, on the other hand, can generally be more accurately performed than determinations of absolute weights per unit area, as some of the experimental errors disappear, such as the error of the determination of α , and, to a considerable extent, the error related to the calibration curve.

CHAPTER 9

Applications

Utilizing the procedures described in Chapter 8 the spatial resolving power of the quantitative microradiographic technique was tested on *Diatomacée*. Fig. 34 shows a microradiogram of a *Diatomacé*, registered at 1.0 kV across the roentgen tube, and secondarily magnified 1500 times. The thin walls of the transversal channels in this organism are clearly seen in the microradiogram, and the distance between the walls is about $1\ \mu$. The spatial resolving power can be estimated at about $0.4\ \mu$.

Thin sections of soft tissues with a regular structure are good test objects both for the spatial and the quantitative resolving power of the microradiographic technique. A $3\ \mu$ thick section of a *striated muscle* from *Chironomus* is shown in the microradiogram in Fig. 35, registered at a voltage of 1.0 kV and secondarily magnified 1500 times. Anisotropic bands with a high total dry weight and a width of between 0.5 and $0.7\ \mu$ alternate with somewhat broader isotropic bands with a width of between 1.3 and $1.5\ \mu$ which have a lower dry weight. The ratio between the dry weights per unit volume in the anisotropic and isotropic bands was $(1.41 \pm 0.05):1$ in a series of 10

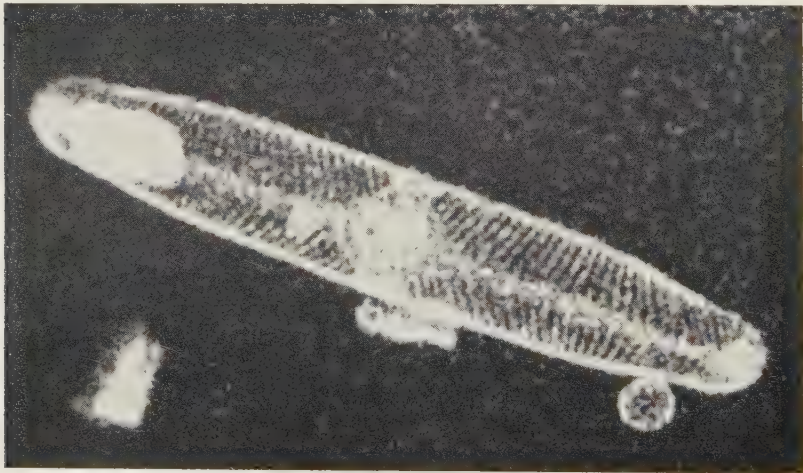


Fig. 34. Microradiogram, $\times 1500$, of a *Diatomacé*, registered at 1.0 kV.

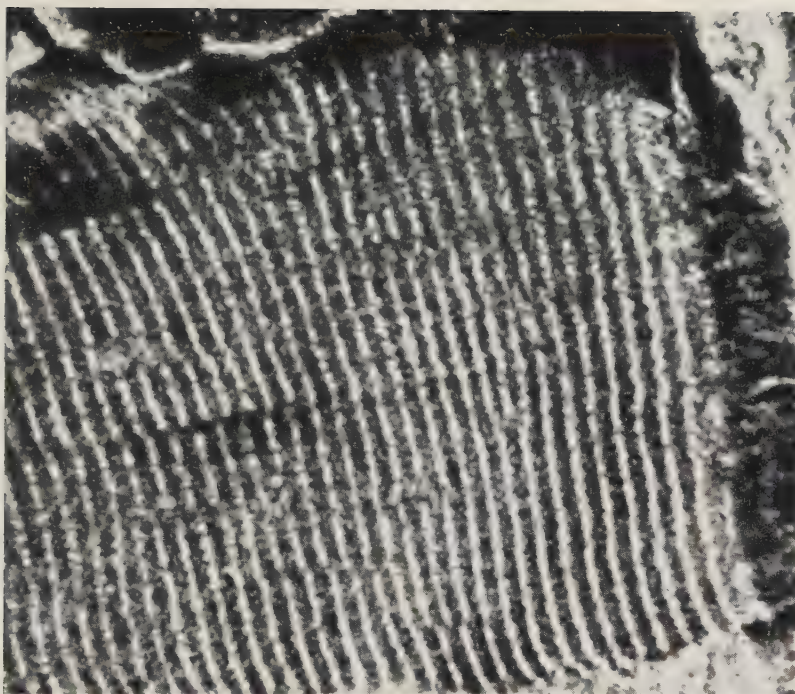


Fig. 35. Microradiogram, $\times 1500$, of a $3\ \mu$ thick section of a formalin fixed striated muscle of *Chironomus*, registered at 1.0 kV.

determinations, including the biologic variation. The photometric measurements were performed on areas with a size of about $1.5\ \mu^2$ in order to resolve the periodic band structure.

In most biologic material the cells and cytologic structures are distinguished by a varying total dry weight per unit volume owing to differences in the content of water in the fresh tissue. When different quantitative cytochemical analyses are performed on sections of solid tissues, such variations of the dry weight have a great influence upon the analytic results. Cytochemical analyses, expressed per unit area, reflect, to a certain extent, these variations of the total dry weight. If the analyses are related to the dry weight, the results are obtained per unit weight, independent of shrinkage effects, and can be compared with microchemical analyses.

The medullary cells of the *adrenals* have a high lipid and water content. Quantitative measurements of the total dry weight in thin sections of frozen-dried normal rat adrenals, deparaffinized in chloroform, revealed a higher



Fig. 36. Microradiogram, $\times 675$, of a $7\ \mu$ thick section of frozen-dried adrenal of the rat, registered at 1.5 kV.

dry weight of the cortical cells than of the medullary cells (Fig. 36). The ratio between the dry weights per unit volume of the two types of cells was 4.0:1 with a relative standard error of the mean of ± 12 per cent, including the biologic variation. The value is based on 20 photometric measurements in each of the two layers of the adrenal. As most of the lipids are dissolved during the deparaffinizing, the ratio between the dry weights is mainly a measure of the different concentrations of proteins in the cortical and medullary cells.

The structural changes of the walls of the arteries during the process of arteriosclerosis are accompanied by a successive increase of the dry weight. Thus, in the kidney parenchyma in *nephrosclerosis*, the minute vessels are distinguished by a rather high dry weight (Fig. 37). The hyalinization of the afferent glomerular arterioles, characteristic of the kidney in *hypertensive arteriolar sclerosis*, corresponds to a marked increase of the dry weight of the walls of these vessels (Fig. 38). Within the glomerulus the thickening of the capillary walls is demonstrable, and is accompanied by an increased dry weight as compared with normal capillaries.

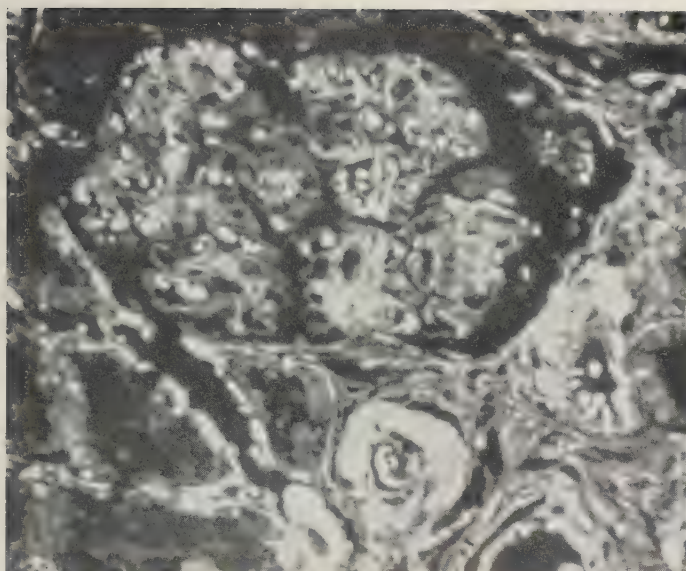


Fig. 37. Microradiogram, $\times 300$, of a $3\ \mu$ thick section of formalin fixed human kidney in nephrosclerosis, registered at 1.5 kV.

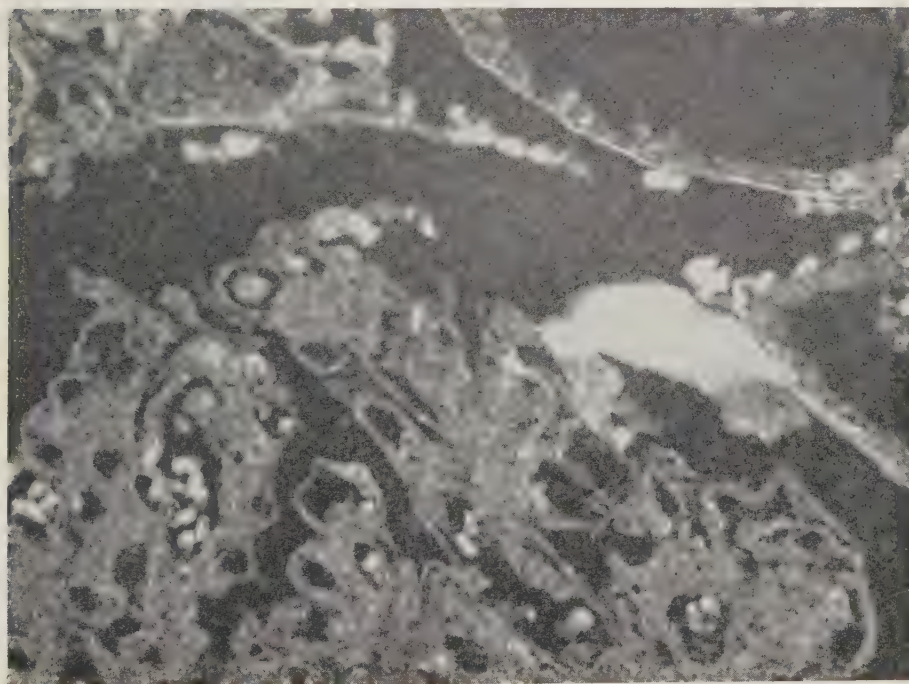


Fig. 38. Microradiogram, $\times 675$, of a $3\ \mu$ thick section of formalin fixed human kidney in hypertensive arteriolar sclerosis, registered at 1.5 kV.

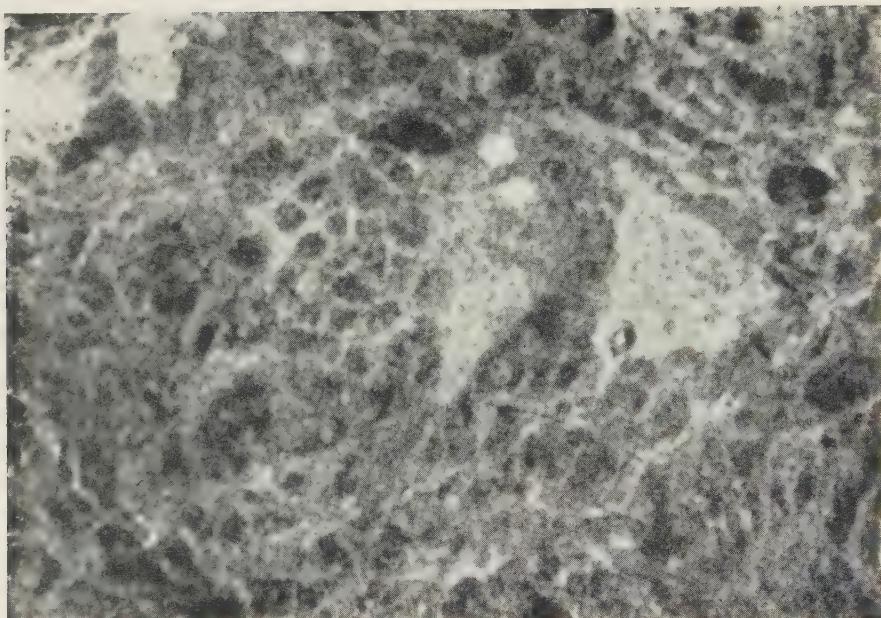


Fig. 39. Microradiogram, $\times 675$, of a $5\ \mu$ thick section of formalin fixed human foreign body granuloma, registered at 1.5 kV.

The total resolving power, obtained in the primary microradiograms in the present investigation, makes it possible to determine the dry weight per unit area in individual cellular structures in thin sections of solid tissues. Thus, in a *foreign body granuloma*, the cytoplasm of the giant cells had a higher absorption of soft continuous roentgen radiation than other cells in the granulomatous tissue (Fig. 39). This indicates a rather high dry weight of the giant cells, caused by a high concentration of organic material, mainly proteins, in these cells. Foreign body inclusions within the giant cells appear as corpuscles with an extremely high absorption of roentgen radiation and can therefore be easily recognized in the microradiograms.

Molluscum contagiosum represents a contagious virus disease of the human skin, where cells in different stages of virus reproduction can be found in the different layers of the virus-infected epidermis. Thus, from the basal to the superficial spinous layers, the cells are increasingly occupied by the virus. A successive increase of the dry weight of the individual cells towards the superficial layers is simultaneously found (Fig. 40). The ratios between the dry weights per unit volume in cells on three different levels in the

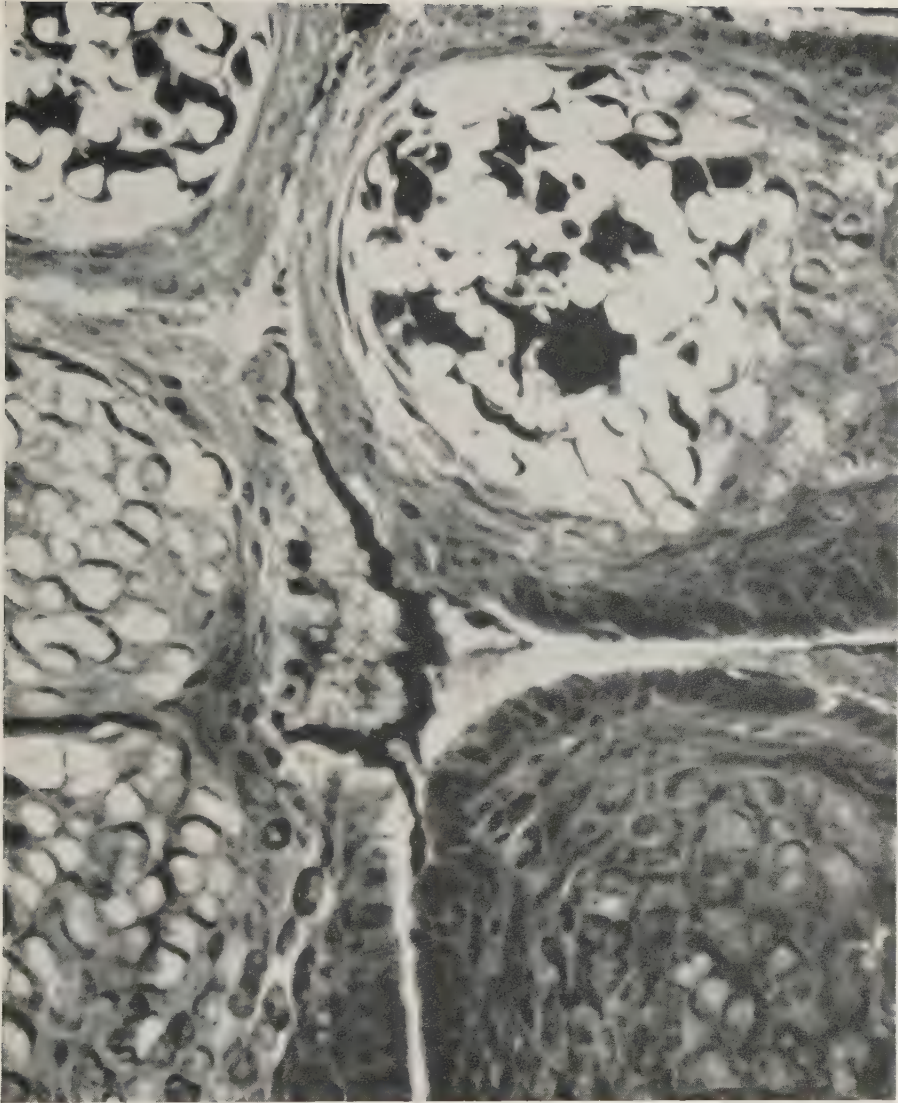


Fig. 40. Microradiogram, $\times 265$, of a $5\ \mu$ thick section of formalin fixed human epidermis with *Molluscum contagiosum*, registered at 1.5 kV.

same microtome sections were determined from the ratios between the foil equivalents, u_x in equation (74), each equivalent determined from 20 photometric measurements. In the basal and lower spinous layers, where no morphological changes indicated the presence of virus, the foil equivalent was

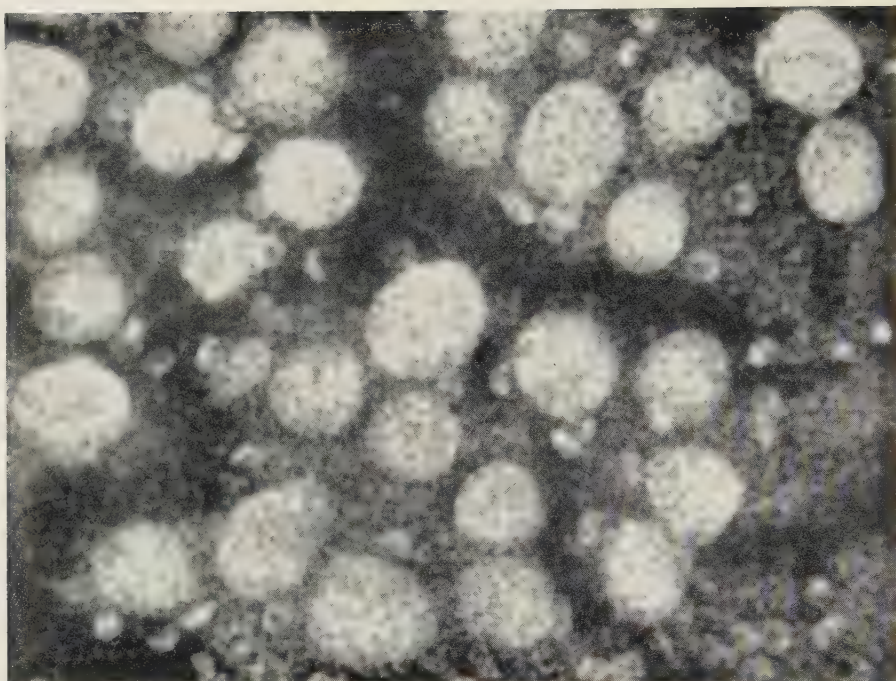


Fig. 41. Microradiogram, $\times 1050$, of frozen-dried ascites tumor cells, registered at 2.0 kV.

1.06 ± 0.05 . In the middle portion of the spinous layer the foil equivalent of the virus-infected cells was 1.735 ± 0.05 . The corresponding value for the superficial cells was 3.79 ± 0.08 . The ratios between the dry weights per unit volume of virus-infected cells in these two layers and the cells in the basal and lower spinous layers were $(1.64 \pm 0.09) : 1$ and $(3.58 \pm 0.19) : 1$, respectively. The standard errors of the ratios were calculated according to equation (72). Thus the intracellular reproduction of the virus in these mammalian cells is accompanied by a more than threefold increase of the concentration of organic material, as compared with normal spinous or basal cells.

One of the main purposes of quantitative cytochemistry is determinations of total amounts of different substances per cell. However, in sections of solid tissues, such determinations can only rarely be performed, because of the difficulties of determining the thickness of the absorbing layer and the volumes of individual cells from a series of thin microtome sections of the tissue.

Table XV. Total dry weight of individual ascites tumor cells (MC1M)

Cell No.	u_c	a μ	m_c $\mu\mu g$	Cell No.	u_c	a μ	m_c $\mu\mu g$
1	0.97	17.9	377	11	1.015	19.0	444
2	0.74	18.1	293	12	1.67	17.3	606
3	0.97	18.8	416	13	0.85	19.1	376
4	0.925	17.8	355	14	1.10	16.8	377
5	1.45	17.7	551	15	0.95	16.9	329
6	1.10	15.8	333	16	0.995	16.3	321
7	0.95	16.3	306	17	1.10	17.3	400
8	1.54	15.7	461	18	0.85	18.0	334
9	1.49	17.0	522	19	1.22	17.5	454
10	1.22	16.1	384	20	0.70	17.7	266

Mean of total dry weight per cell = $(395 \pm 20)\mu\mu g$.

Fresh smears of some *ascites tumors*, kindly provided by Dr. G. KLEIN and Dr. E. KLEIN, were frozen-dried and prepared for roentgen absorption analyses according to the technique described in Chapter 8: B. Fig. 41 shows a microradiogram of ascites tumor cells, registered at 2.0 kV and secondarily magnified 1050 times. In these frozen-dried specimens the nuclei have the same total dry weight per unit volume as the cytoplasm of the cells. The foil equivalent u_c was measured in the central portion in the roentgen absorption image of each cell, and the corresponding total dry weight per unit area was thus $1.32 u_c w$ according to equation (74). The aperture in front of the photomultiplier tube corresponded to an area of about $1.5 \mu^2$ in the microradiogram. The cells were considered to have an approximately spherical shape, and the mean diameter for each cell ($a \mu$) was measured in high power photo-micrographs of the primary microradiograms. The weight per unit volume (μ^3) was then $1.32 u_c w a^{-1}$, where $w = 1.755 \mu\mu g \cdot \mu^{-2}$. The total dry weight m_c per cell was calculated according to

$$m_c = 1.32 u_c w \frac{\pi a^2}{6} \dots\dots\dots (75).$$

In table XV 20 individual measurements of the total dry weight per cell are presented. The mean value of the dry weight per cell was $(3.95 \pm 0.20)10^{-10}$ g, in good agreement with the values obtained from microchemical analyses on large cell populations.

These few applications demonstrate that the quantitative microradiographic procedure can be utilized to determine the total dry weight of organic material in volumes as small as $5 \mu^3$. The spatial resolving power is about 0.4 or 0.5μ and the quantitative resolving power is sufficient for the determination of the amount of organic material in biologic objects with a thickness of about 2μ . Since no structural or chemical changes of the specimens occur during the quantitative microradiographic measurements, subsequent analyses, utilizing other cytochemical methods, can be correlated with the total dry weight of cytologic structures.

PART III

Elementary Analyses of Small Biologic Objects by Quantitative Roentgen Absorption Spectrophotometry

CHAPTER 10

Survey of Quantitative Elementary Analyses by Roentgen Ray Methods with Special Reference to Biologic Applications

A. Quantitative Roentgen Ray Fluorescence Analyses

Because of the nature of the characteristic roentgen spectra, which was discussed in Chapter 1: B, *roentgen ray fluorescence analyses* give qualitative and, with special techniques, quantitative information of the elementary composition of a chemical compound or a mixture [199]. The characteristic roentgen spectrum of the element to be analysed can be generated by electron bombardment, i.e. by *primary excitation*, or by roentgen radiation, i.e. by *secondary excitation*. When utilizing the first procedure the specimen forms a part of the anode of the roentgen tube and must therefore be resistant to high temperatures, which is not necessary when applying secondary excitation. This latter method is therefore most suitable for biologic material. The fluorescence yield, however, is small for elements with low atomic number, as discussed in Chapter 2: C (see Table II). This is unfavourable when roentgen ray fluorescence analyses are applied to biologic objects which are composed mainly of elements with low atomic numbers. The sensitivity of these methods and the possibilities to apply them on biologic material have been discussed by ENGSTRÖM [29].

Geiger counter spectrometers for roentgen ray fluorescence analyses have been constructed [196, 185], and such equipment has been utilized for analyses of tetraethyllead in gasoline [188], of uranium [187], and of different other metals [186, 208]. These methods are, however, unsuitable for cytochemical purposes because of the difficulties of obtaining a sufficient spatial resolving power. For metallographic purposes an equipment for roentgen ray fluorescence analyses of volumes of about $1 \mu^3$ has been constructed [191], where a fine beam of electrons is generated in an electron microscope, and the characteristic roentgen spectra of the elements composing the specimen are primarily excited and registered by a Geiger counter roentgen ray spectrophotometer.

A review of recent developments in roentgen ray fluorescence analyses has been given by CLARK [15, pp. 147—159]. However, no cytochemical investigations utilizing these methods, have been published.

B. Quantitative Elementary Analyses of Chemical Compounds or Mixtures by Röntgen Absorption Spectrophotometry

GLOCKER and FROHNMEYER [197] introduced elementary analyses by means of *roentgen absorption spectrophotometry*, utilizing an absorption edge of the element to be analysed. The characteristics of these absorption edges have been discussed in Chapter 2: D. From measurements of the absorption of monochromatic roentgen radiation in the specimen at wavelengths close to and on both sides of an absorption edge, the total amount of the corresponding element could be determined, independent of the chemical combination of the element and of the presence of other elements. When determining barium in a solution of barium chloride the experimental error was about ± 10 per cent. The monochromatic roentgen radiation was obtained from a *continuous roentgen spectrum*, generated in a commercial roentgen tube, and diffracted by a plane crystal (see Chapter 3: B). The intensities were measured by a photographic-photometric procedure.

It has been stated that elements with atomic numbers lower than 42 can not be analysed, utilizing this method. The K absorption edges of these elements are situated at wavelengths longer than about 0.65 kX.U., and insufficient intensities of monochromatic roentgen radiation should be obtained at longer wavelengths [15, p. 160]. It will be shown in the present work, however, that calcium ($Z=20$, K absorption edge at 3.064 kX.U.) can be analysed by a photographic-photometric procedure, when the continuous roentgen spectrum from an efficient roentgen tube is diffracted by a bent crystal.

In order to obtain higher intensities of monochromatic roentgen radiation for the elementary analyses by roentgen absorption measurements, MOXNES [209, 210] utilized the *characteristic roentgen spectrum* of the anode material, which was chosen to give roentgen emission lines with wavelengths on both sides of the absorption edge of the element to be analysed. Analyses of zinc and iron were performed, utilizing a photographic-photometric procedure, but the accuracy was rather low.

A further improvement of the roentgen absorption method was made by

VOGES [211], utilizing "the filter difference method" according to Küstner (see Chapter 3:A) to obtain high intensities of monochromatic roentgen radiation and an ionization chamber to measure the roentgen intensities. Two or three measurements with monochromatic roentgen radiation were made at different wavelengths on each side of the absorption edge of the element under investigation. As an approximate linear relationship holds between the logarithms of the mass absorption coefficient and the wavelength of the roentgen radiation (see Fig. 4), the magnitude of the absorption difference at the edge could be obtained from extrapolation. Accurate elementary analyses of copper, molybdenum, silver, and tin were made with an error of only about ± 3 per cent.

LAURILA [201] applied the original method of Glocker and Frohnmayer to analyse bromine and rubidium, utilizing an ionization roentgen ray spectrophotometer. During the last few years roentgen absorption methods have become of increasing importance for the chemical analyses of different elements or compounds, i.e. the determination of tetraethyllead in gasoline. The scintillation counter and the different counters based upon the ionizing effect of roentgen radiation (see Chapter 4:B,C) have been utilized to register the roentgen intensities. For reviews of roentgen ray absorption methods in chemical analyses reference should be made to LIEBHAFSKY [202—207].

C. Elementary Analyses of Biologic Objects by Quantitative Roentgen Absorption Spectrophotometry

In the different publications, discussed in the previous section, the elementary analyses were performed on rather large areas. SIEVERT [177] suggested a combination of a microradiographic procedure and the utilization of the absorption edges of different elements to obtain a selective increase of the contrast in the roentgen absorption image, making possible an analysis of the distribution of some elements in small objects.

ENGSTRÖM [29] was the first to apply roentgen absorption methods to histochemical elementary analyses *in situ* on microtome sections of biologic objects. Characteristic roentgen emission lines of different anode materials, and in some cases secondarily excited emission lines, were utilized. The roentgen tubes were continuously evacuated, and the individual emission lines were separated by diffraction by plane crystals. Microchemical determinations

were made of zinc and iron in solutions in micro-cuvettes, and of iodine in solutions of potassium iodide sucked up on filtering paper. Utilizing a photographic-photometric procedure, the errors of the roentgen absorption analyses were about ± 10 per cent.

The histochemical elementary analyses were performed on calcified tissues, and the microradiograms were registered on Printon films with an estimated spatial resolving power of 15μ [29]. The amount of calcium in calcifications in coronary vessels, and the amounts of phosphorus and calcium in dentine cells were determined with an estimated error of ± 10 per cent. One single histochemical elementary analysis has been performed on soft tissues by roentgen absorption analyses, *viz.* a determination of the amount of sulphur in epithelium [194], but the results were unreliable [171].

Only low intensities of monochromatic roentgen radiation can be obtained when utilizing a plane crystal (see Chapter 3), especially at wavelengths longer than about 3 kX.U. Therefore, plane crystals are unsuitable for elementary analyses on a cytochemical scale, as such determinations require extremely fine-grained photographic emulsions which are about 1000 times less sensitive to roentgen radiation than Printon film [143].

The Theoretical Basis for Elementary Analyses by Roentgen Absorption Spectrophotometry

A. The Principle of the Method

The principle for elementary analysis of an element y in a chemical compound or mixture by roentgen absorption spectrophotometry is illustrated in Fig. 42. The continuous curve gives the mass absorption coefficient of the element y to be investigated as a function of the wavelength of the roentgen radiation, and the dash and dotted curve represents the corresponding function of the remaining portion r of the specimen. If monochromatic roentgen radiation with the wavelength λ_1 and the intensity I_{01} is incident on the specimen, the transmitted roentgen intensity I_{11} is calculated according to equation (23)

$$I_{11} = I_{01} e^{-\left(\frac{\mu_1}{\rho}\right)_y m_y - \left(\frac{\mu_1}{\rho}\right)_r m_r} \dots\dots\dots (76),$$

where m_y and m_r are the weights per unit area ($\text{g}\cdot\text{cm}^{-2}$) of y and r respectively. For roentgen radiation with the wavelength λ_2 the analogous equation is obtained

$$I_{12} = I_{02} e^{-\left(\frac{\mu_2}{\rho}\right)_y m_y - \left(\frac{\mu_2}{\rho}\right)_r m_r} \dots\dots\dots (76 \text{ a}).$$

m_r is eliminated in equations (76) and (76 a), and the expression is solved for m_y :

$$m_y = \frac{\ln \frac{I_{01}}{I_{11}} - \left[\left(\frac{\mu_1}{\rho}\right)_r / \left(\frac{\mu_2}{\rho}\right)_r \right] \ln \frac{I_{02}}{I_{12}}}{\left(\frac{\mu_1}{\rho}\right)_y - \left(\frac{\mu_2}{\rho}\right)_y \left[\left(\frac{\mu_1}{\rho}\right)_r / \left(\frac{\mu_2}{\rho}\right)_r \right]} \dots\dots\dots (77),$$

which is the expression deduced by ENGSTRÖM [29].

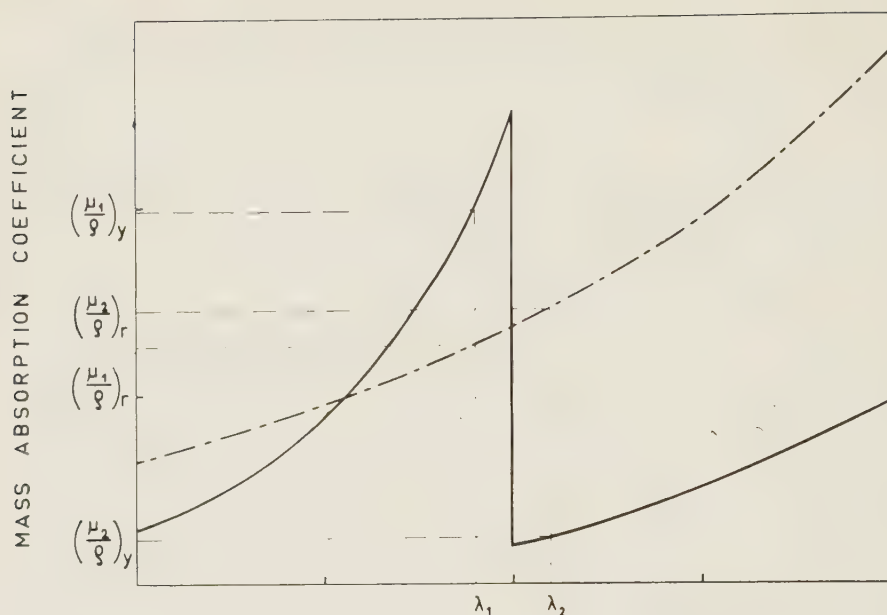


Fig. 42. Schematic illustration of the principle for elementary analysis.

In the present work equation (77) will only be applied to analyses performed at wavelengths longer than about 2.5 kX.U., and the mass scattering coefficient can therefore be neglected (see Chapter 2:C). Some different absorption formulae were discussed in Chapter 2:E, and according to equation (27) the mass photoelectric absorption coefficient for a given element is proportional to λ^u , where u has a value of about 3.

However, more accurate results are obtained if equation (31) is utilized. The difference between the mass absorption coefficients of an average animal protein and nitrogen is very small, as can be seen from Tables IV and VII. Therefore, for soft tissues the "average atomic number" can be estimated at about 7. From Jönsson's tables the value of $f(Z\lambda)$ in equation (31) can then be calculated at different wavelengths. For other tissues it may be necessary to calculate approximate, composite mass absorption

coefficients. Hence $\left(\frac{\mu_1}{\rho}\right)_r / \left(\frac{\mu_2}{\rho}\right)_r$ can be replaced by $\left(\frac{\lambda_1}{\lambda_2}\right)^{f(Z\lambda_{1,2})}$ in most

cases, provided λ_1 and λ_2 are situated close to the absorption edge. Equation (77) is modified to the following expression [29] :

$$m_y = \frac{\ln \frac{I_{01}}{I_{11}} - \left(\frac{\lambda_1}{\lambda_2} \right)^{f(Z\lambda_{1,2})} \cdot \ln \frac{I_{02}}{I_{12}}}{\left(\frac{\mu_1}{\rho} \right)_y - \left(\frac{\mu_2}{\rho} \right)_y \left(\frac{\lambda_1}{\lambda_2} \right)^{f(Z\lambda_{1,2})}} \dots \dots \dots (78).$$

In this equation the denominator is termed $f(\mu)$.

When the elementary analyses are performed according to GLOCKER and FROHNMEYER [197], $\lambda_1 = \lambda_2$, and $I_{01} = I_{02}$, and hence

$$m_y = \frac{\ln \frac{I_{12}}{I_{11}}}{\left(\frac{\mu_1}{\rho} \right)_y - \left(\frac{\mu_2}{\rho} \right)_y} \dots \dots \dots (79).$$

When the wavelengths of the utilized monochromatic roentgen lines are situated at a distance from the absorption edge, the method of extrapolation according to VOGES [211] must be utilized. For a discussion of this procedure reference should be made to ENGSTRÖM [29].

B. The Accuracy and the Sensitivity of the Method

In equations (78) and (79) the different intensities of roentgen radiation are measured experimentally. The standard error ε_y of m_y in equation (78) can be calculated according to equation (67) :

$$\varepsilon_y^2 [f(\mu)]^2 = \left(\frac{dI_{01}}{I_{01}} \right)^2 + \left(\frac{dI_{11}}{I_{11}} \right)^2 + \left(\frac{\lambda_1}{\lambda_2} \right)^{2f(Z\lambda_{1,2})} \left[\left(\frac{dI_{02}}{I_{02}} \right)^2 + \left(\frac{dI_{12}}{I_{12}} \right)^2 \right] \dots \dots \dots (78 a),$$

where dI_{01} , dI_{11} , dI_{02} , and dI_{12} are the standard errors of the different intensities. If the experimental determinations of the intensities have a coefficient of variation of $\pm 100 q$ per cent, equation (78 a) takes the form

$$\varepsilon_y = \pm \frac{q\sqrt{2}}{f(\mu)} \sqrt{1 + \left(\frac{\lambda_1}{\lambda_2} \right)^{2f(Z\lambda_{1,2})}} \dots \dots \dots (78 b).$$

For the standard error ε_y of m_y in equation (79) the analogous equations are obtained:

$$\varepsilon_y^2 \left[\left(\frac{\mu_1}{\varrho} \right)_y - \left(\frac{\mu_2}{\varrho} \right)_y \right]^2 = \left(\frac{dI_{12}}{I_{12}} \right)^2 + \left(\frac{dI_{11}}{I_{11}} \right)^2 \dots\dots\dots (79 \text{ a}),$$

and

$$\varepsilon_y = \pm \frac{q \sqrt{2}}{\left(\frac{\mu_1}{\varrho} \right)_y - \left(\frac{\mu_2}{\varrho} \right)_y} \dots\dots\dots (79 \text{ b}).$$

Utilizing monochromatic roentgen radiation at wavelengths close to the absorption edge of the analysed element, m_y , calculated according to equation (78), has thus a standard error ε_y about $\sqrt{2}$ times greater than the corresponding error of m_y , calculated according to equation (79), provided the precision of the measurements of the intensities is the same in the two cases.

According to equation (79) the amount m_y is inversely proportional to the difference $\left(\frac{\mu_1}{\varrho} \right)_y - \left(\frac{\mu_2}{\varrho} \right)_y$, and for a given minimum value of $\ln I_{12}/I_{11}$ this difference determines the minimum measurable amount per unit area of the element. The different empirical formulae, expressing the magnitude of the absorption edges and the values of the mass absorption coefficients, have been discussed in Chapter 2: E, where references to the physical literature are given. The absolute difference between the mass absorption coefficients at an absorption edge shows a greater increase with decreasing atomic number of the absorbing element than the magnitude of the edges, plotted in Fig. 5 and Fig. 6. For a given element the absolute difference between the mass absorption coefficients is smaller at the K edge than at the L_{III} edge, and the difference at the M_V edge is still greater. A diagram of these differences as a function of the atomic number of the absorbing element has been published by ENGSTRÖM [29].

In Table XVI the minimum value of m_y ($\mu\mu g \cdot \mu^{-2}$) has been calculated according to equation (79 b) for some different values of the difference $\left(\frac{\mu_1}{\varrho} \right)_y - \left(\frac{\mu_2}{\varrho} \right)_y$ and of the experimental error q . The allowable relative error ε_y/m_y of the elementary analyses has been fixed at ± 0.05 . With the guidance of the values in the table and from microchemical determinations of average concentrations of different elements in tissues, suitable thicknesses

Table XVI. The smallest measurable amount of an element

$\left(\frac{\mu_1}{\rho}\right)_y - \left(\frac{\mu_2}{\rho}\right)_y$	Minimum values of m_y ($\mu\mu g \cdot \mu^{-2}$) $\varepsilon_y/m_y = \pm 0.05$		
	q = 0.005	q = 0.01	q = 0.02
100	14.14	28.28	56.56
200	7.07	14.14	28.28
500	2.83	5.66	11.31
1000	1.41	2.83	5.66
2000	0.71	1.41	2.83
5000	0.28	0.57	1.13
10000	0.14	0.28	0.57
20000	0.07	0.14	0.28

of the biologic objects can be selected. ENGSTRÖM [29, 192, 193] has discussed the possibilities of performing cytochemical elementary analyses in thin sections of tissues by roentgen absorption spectrophotometry. In sections with a thickness of between 1 and 10 μ , carbon, nitrogen, and oxygen are present in amounts high enough for elementary analyses by roentgen absorption spectrophotometry. Calcium and phosphorus in calcified tissues can be analysed in specimens with a similar thickness. In sections of soft tissues with a thickness of about 20 μ , sulphur and phosphorus may also be determined.

The systematic errors consist of the *uncertainty of the mass absorption coefficients and of the exponent $f(Z\lambda_{1,2})$* , which has been discussed previously [29]. If the exponent is calculated according to equation (31), the error will be small, provided λ_1 and λ_2 are situated close to the absorption edge of the element under investigation.

The systematic error introduced by the uncertainty of the difference $\left(\frac{\mu_1}{\rho}\right)_y - \left(\frac{\mu_2}{\rho}\right)_y$ is much more important and varies from element to element. For elements with $Z < 10$ accurate determinations of this difference are difficult to perform, as only low intensities of monochromatic roentgen radiation can be generated in the extremely soft roentgen region.

In Chapter 2:D the fine structure at the absorption edges was discussed.

These variations of the values of the mass absorption coefficients may influence upon the elementary analyses, if the measurements are made at wavelengths in the immediate proximity of the utilized absorption edge. When performing cytochemical elementary analyses, however, a roentgen ray spectrophotometer with a low resolving power must be used to obtain *high* intensities of relatively monochromatic roentgen radiation. The effect of a fine structure will therefore be negligible at most cytochemical elementary analyses by roentgen absorption spectrophotometry.

CHAPTER 12

The High Vacuum Roentgen Ray Spectrophotometer

A. The Choice of Experimental Apparatus for Elementary Analyses

The absorption edges of elements of biologic interest are situated in the soft roentgen region at wavelengths longer than about 2.5 kX.U. Furthermore, in order to obtain a maximum sensitivity in the elementary analyses, the difference between the mass absorption coefficients shall be as great as possible at the utilized absorption limit of the element. However, for the elements with low atomic numbers, the K absorption edge is generally the only suitable limit, as the wavelengths of the L absorption edges are situated in the extremely soft roentgen region, where monochromatic roentgen radiation can be generated only with low intensities.

The different methods of obtaining monochromatic roentgen radiation have been discussed in Chapter 3, with special reference to procedures suitable in the soft roentgen region. A high vacuum roentgen ray spectrophotometer with bent crystal according to Johann (see Chapter 3:C) was considered to have the greatest advantages. With such an equipment high intensities of monochromatic roentgen radiation can be obtained within wide limits of wavelengths.

For elementary analyses on a cytochemical scale by means of roentgen absorption spectrophotometry, the intensity of the roentgen radiation in the absorption image of the specimen must be registered on a fine-grained photographic emulsion, which was discussed in Chapter 4. As these emulsions are extremely insensitive to radiant energy, the resolving power of the diffracting system of the spectrophotometer must be kept as low as possible in order to obtain a maximum energy of monochromatic roentgen radiation.

B. The Principle of the Spectrophotometer

The diffraction and the focusing effect of a bent crystal according to Johann were described in Chapter 3:C (see Fig. 13). The principle of the bent crystal spectrophotometer, designed for elementary analyses, is shown

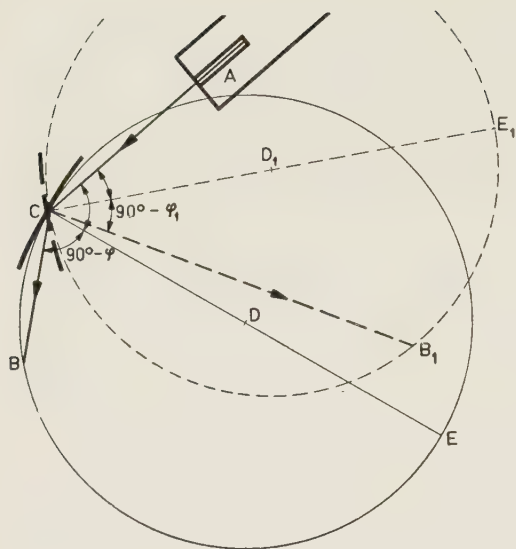


Fig. 43. The principle of the bent crystal spectrophotometer.

in Fig. 43. The anode A has a fixed position, and the distance AC between the focal spot of the roentgen tube and the axis of rotation of the bent crystal is constant. The diffracted monochromatic beam with the glancing angle φ is focused on the Rowland circle at B, and a corresponding beam with the glancing angle φ_1 is focused at B₁. The distance CB is $R \sin \varphi$, where R is the radius of curvature of the bent crystal.

C. The Body of the Spectrophotometer

The body of the high vacuum spectrometer consists of a metal box ($750 \times 445 \times 200$ mm) made of forge iron (Fig. 44). The walls and the bottom have a thickness of 25 mm and are welded together. The iron lid, strengthened by welded profiles, has a thickness of 15 mm, and the vacuum tightening against the plane upper edges of the walls consists of a plane rubber gasket. The lid is lifted by a pulley block from an overhead track. The roentgen tube (36) is connected vacuum-tight to the body of the spectrometer by means of a plane rubber gasket. It is of the same construction as the roentgen tube for total dry weight determinations, described in Chapter 7. The horizontal projection of the axis of the anode forms an angle of 40° to the long walls of the metal box, and the axis itself an angle of 8° to the horizontal plane through the spectrometer. The roentgen radiation is admitted to the

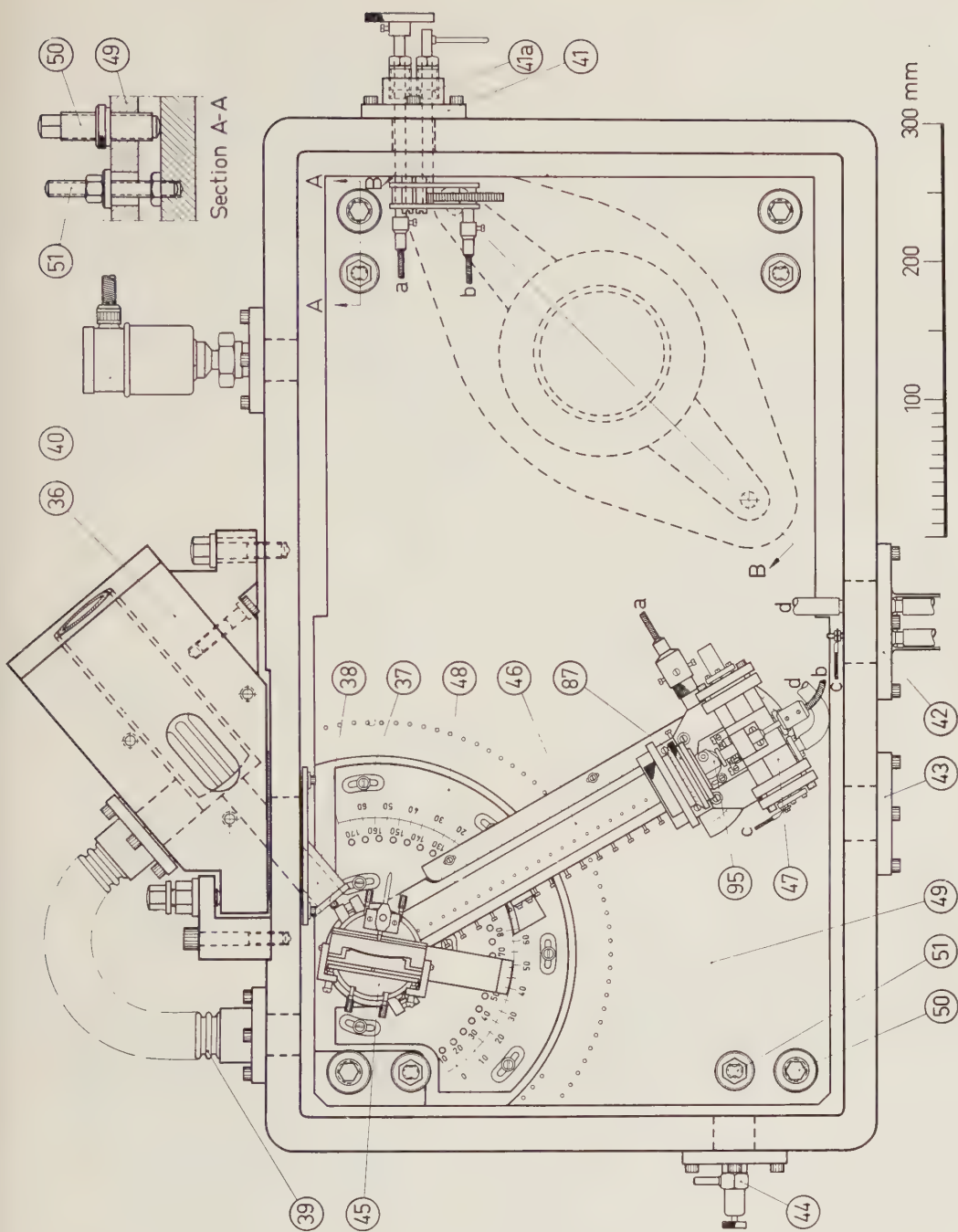


Fig. 44. Horizontal projection of the spectrophotometer. The cathode and the insulator of the anode removed and the anode cut.

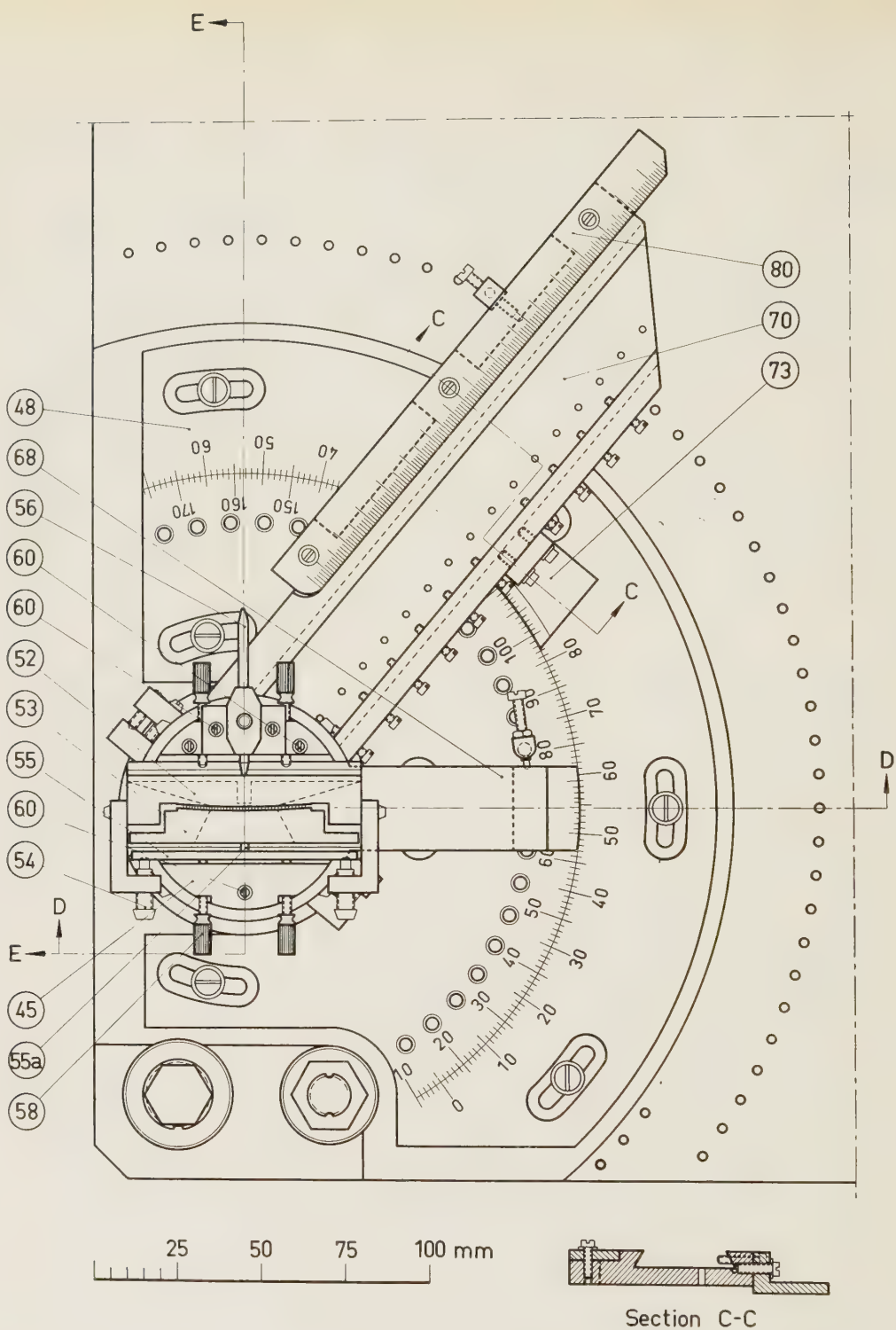


Fig. 45. Horizontal projection of the crystal holder, the crystal table and the short arm for the registering system.

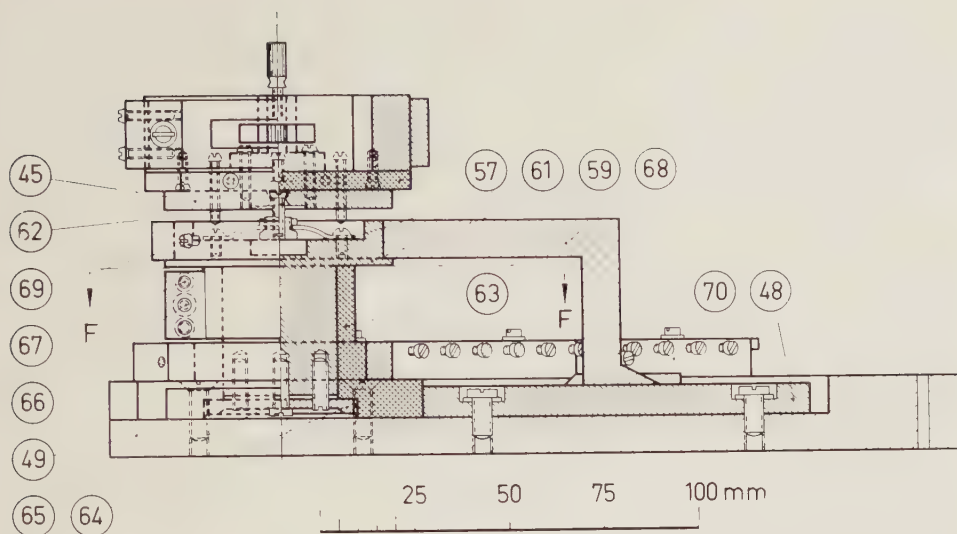


Fig. 46. Section D—D in Fig. 45.

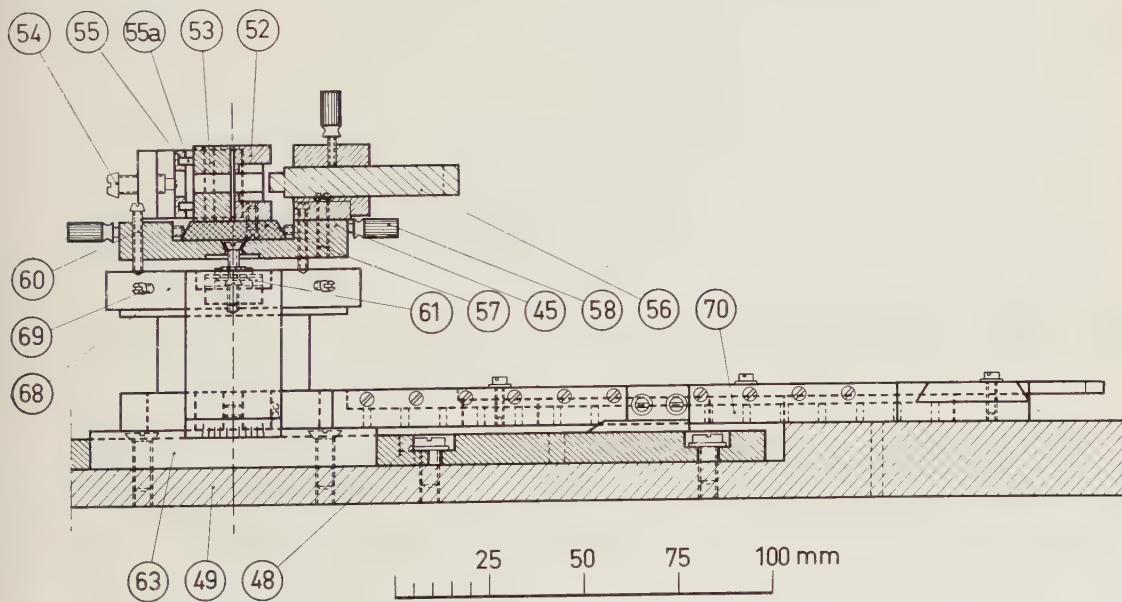


Fig. 47. Section E—E in Fig. 45. The arm parallel with the section.

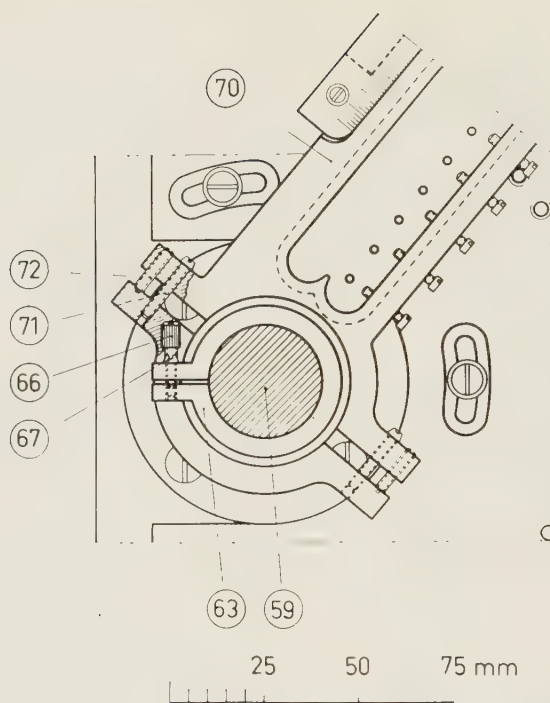


Fig. 48. Section F—F in Fig. 46.

spectrometer through a thin aluminium foil with a weight of about $0.5 \text{ mg} \cdot \text{cm}^{-2}$, mounted over a rectangular hole ($6 \times 18 \text{ mm}$) in a metal plate (37). This plate is screwed to a special holder (38), attached to the inside of the wall. The roentgen tube is evacuated simultaneously with the body of the spectrometer through a tombak tube (39).

The body of the spectrometer is provided with inlets for different purposes. A Philips vacuum gauge (40) is connected to the wall. Two axes, transferring rotational movements to the inside of the spectrometer, are attached to the holder (41). Vacuum tightening is secured by two Gaco sealing rings (41 a), permitting rotational movements of the axes. Another plate (42) has inlets for filling the Geiger counter with gas and terminal lugs for the voltage supply to the counter. A reserve inlet (43) and one with a needle valve (44) are also attached to the wall of the spectrometer.

The crystal holder is mounted on the crystal table (45), and the Geiger counter (47) can be displaced along the graduated arm (46). The arm can

be turned in the horizontal plane, and its position is adjusted on the circular scale on the plate (48). This plate is screwed to the bottom plate (49), which can be adjusted both in the horizontal and the vertical direction relative to the body of the spectrometer by means of the screws (50) and (51), demonstrated in the section A—A in Fig. 44.

D. The Crystal Holder and the Crystal Table

Constructional drawings of the crystal holder and the crystal table are shown in Fig. 45—48. The crystal is bent cylindrically between one concave and one convex cylindrically ground surface on the two steel blocks (52) and (53). The crystal holder is constructed according to the principles given by HAGLUND [198]. The pressure from the two screws (54) is evenly distributed on the steel block (53) by means of a thin steel plate (55) provided with two central pins (55 *a*). Rectangular holes (5×20 mm) are milled in the two steel blocks (52) and (53) corresponding to the maximum opening of the crystal. The steel block (52) is grooved thus allowing glancing angles as small as 10° . The opening of the crystal can be limited by means of an edge-slit (56). The steel block (52) is screwed to the steel plate (57), adjustable in the cut groove in the crystal table (45) by means of four screws (58). The crystal table (45) rests upon the inner cylindrical part (59) of the lower portion of the attachment by means of the three screws (60). The pressure of the screws against (59) is adjusted by the leaf-spring plate (61) and the screw (62). By means of the screws (58) and (60) the bent crystal can be adjusted relative to the axis of rotation of the crystal table and to the path of the roentgen beam. The inner cylindrical part (59) is mounted in bearings in the attachment (63), screwed to the bottom plate (49). The inner part (59) is adjusted without play in vertical direction in the attachment (63) by means of the metal plate (64) and the screw (65). The concentric bearing is adjusted by means of the screw (66) and the stop screws (67). The arm (68), provided with a nonius scale to read off the glancing angle, is thread upon and adjustable fastened to the cylindrical, revolving part (59) by means of four screws (69). These are placed diagonally in pairs which makes it possible to fix the arm (68) in two positions with a mutual angle of 90° relative to the revolving part (59). The plate (48) is fastened to the bottom plate (49) by means of seven screws making possible a certain angle of offset angular displacement of the plate.

E. The Crystals

Mica crystals and quartz crystals (from Firm Steeg and Reuter, Bad Homburg) were used in the present work. The size of the crystals was $40 \times 20 \times 0.25$ mm, and the radii of curvature employed were 200 mm and 385 mm. In the quartz crystals the grating spaces 4.246 and 3.336 kX.U. were utilized.

Barium-copper-stearate crystals were used in some model experiments, utilizing extremely soft roentgen radiation. The technique of making the crystals was the one described by BLODGETT [189], and utilized by ANDREWS [183] to make crystals for the purpose of roentgen spectroscopy. The layers of Ba-Cu-stearate crystals were deposited on a cylindrically ground glass surface with a radius of curvature of 407 mm. Lead glass was utilized in the form of rectangular plates ($60 \times 20 \times 10$ mm) which were fixed on a special holder placed on the crystal table (45). In order to avoid the difficulties of depositing the first layer, the clean glass surface was dipped in melted ferristearate, made according to the procedure published by KOENIG [200], and was subsequently polished. The crystal utilized was composed of 200 layers of Ba-Cu-stearate. The value of the grating space is 50.47 Å.U. according to BERNSTEIN [184].

F. The Registering System

The registering system is mounted on either of the two graduated arms (46) or (70), respectively. The short arm (70) is fixed in concentric bearing on the attachment (63) of the crystal holder by means of the screw (71) and the stop screws (72) (Fig. 48). The arm can therefore be turned around the axis of rotation of the crystal holder, and the position can be read off on the circular scale on the plate (48) with the aid of the nonius scale on the plate (73). The specimen holder, mounted on steel plates, can be moved along the arm in a cut groove. Constructional drawings of the photographic registering system are presented in Fig. 49 and 50.

The bottom plate (74) can be displaced along the groove on the arm (70), and its position can be read off on the scale (80) on the arm by means of a nonius scale on the plate (79). The steel plate (75) can be turned around the cylindrical part (76) relative to (74) and is locked by means of the screw (78). The rotation is read off on the circular scale on the steel plate (77)

which is screwed on the plate (74). Another steel plate (81) can be displaced in a groove in the plate (75) by means of the micrometer screw (82), and a pull-off spring (83) (Fig. 51). The top plate (84) can be turned around the cylindrical plate (85) which is screwed to the plate (81). The position of the plate (84) is fixed by a locking screw (86) and can be read off on the circular scale on (81).

The plate (84) serves as attachment for a symmetric slit (87) and for different registering equipment. The opening of the slit can be fixed in horizontal or vertical position by means of the lock ring (88). The slit is placed in the axis of rotation of the plate (84). This axis coincides with the corresponding axis of the plate (75) when the micrometer screw is in the middle position. The arrangement makes it possible to displace the slit opening along the tangent to the Rowland circle, keeping the plane of the slit at right angles to the path of the roentgen beam.

For photographic registering the holder (89) is screwed on the slit (87). The specimen holder, consisting of the parts (90), (91), and (92), is attached to the holder (89) by means of the screw (93). The plate (94), which is fastened behind the slit (87) on the plate (84) serves as attachment for the Geiger counter (47) and for metal parts for total reflection arrangements (95). The scale on the plate (94) is used for the adjustment of the glancing angle at total reflection.

The construction of the Geiger counter is shown in Fig. 51 and 52. The principle described by BROGREN [190] has been utilized. The Geiger counter is made of brass, and the chamber has a length of 80 mm and a diameter of 30 mm. The radiation is admitted to the middle portion of the chamber at right angles to the longitudinal axis, passing through an opening in the plate (97), covered by a thin foil made of formvar. The gables (98) of the Geiger counter are made of plexiglass. The stainless thread (99) has a diameter of 0.05 mm and is stretched by a spring (100). The inlet in the opposite end is made vacuum-tight with picein (101). The thread is centered by means of the brass bolts (102) which are threaded into the gables (98). The effective length of the stainless thread can be adjusted by means of inserting the stainless tubes (103) through the holes in the brass bolts (102). The connection (104) is utilized when filling the Geiger counter with gas. As the formvar foil is very thin, the counter must be evacuated together with the spectrometer, after which it is filled with the gas mixture. The specimens to be analysed are placed in the holder (105) which is provided with two

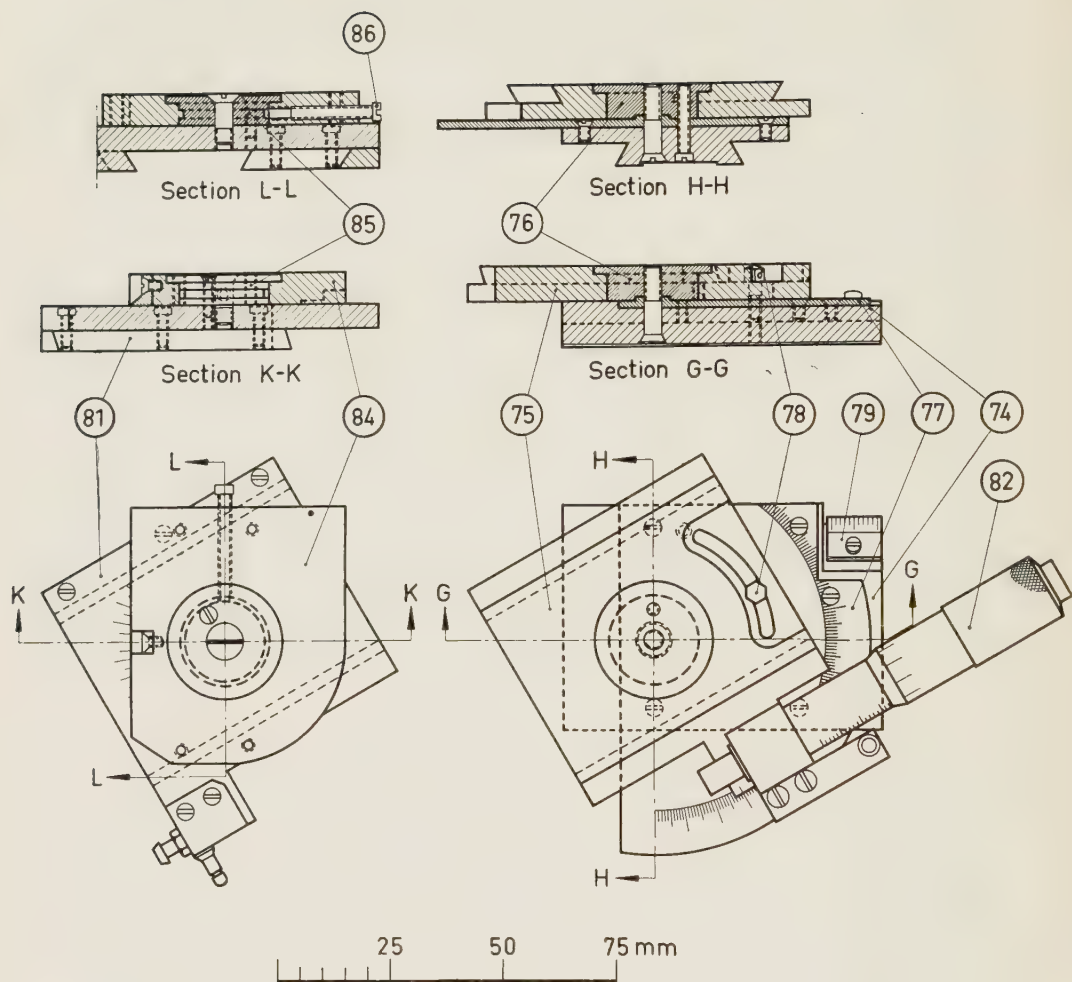


Fig. 49. The lower portion of the registering system.

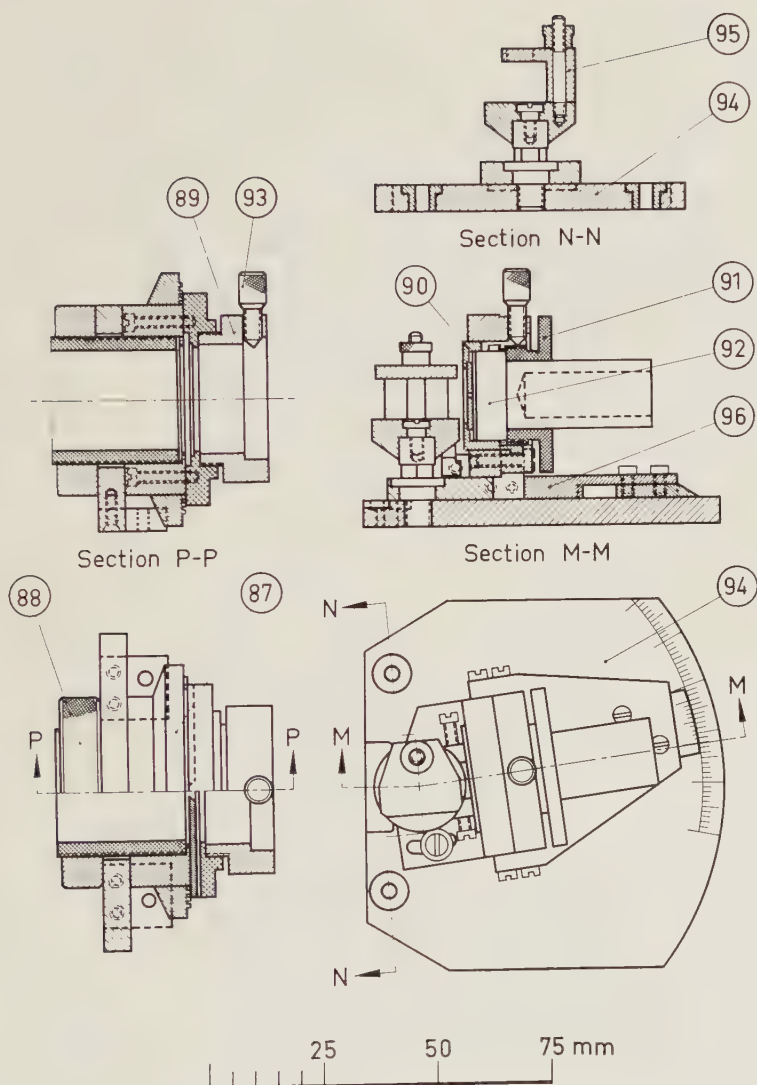


Fig. 50. The upper portion of the registering system with equipment for photographic registering.

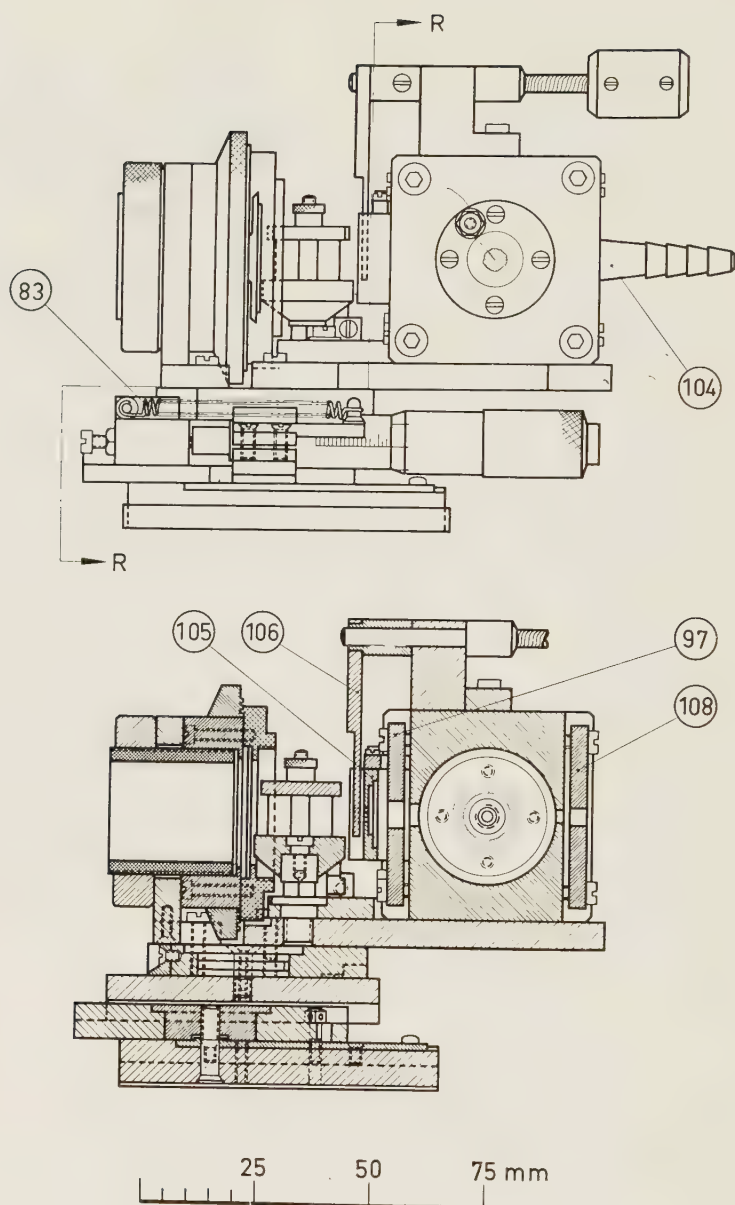


Fig. 51. Side view of the registering system with the Geiger counter.

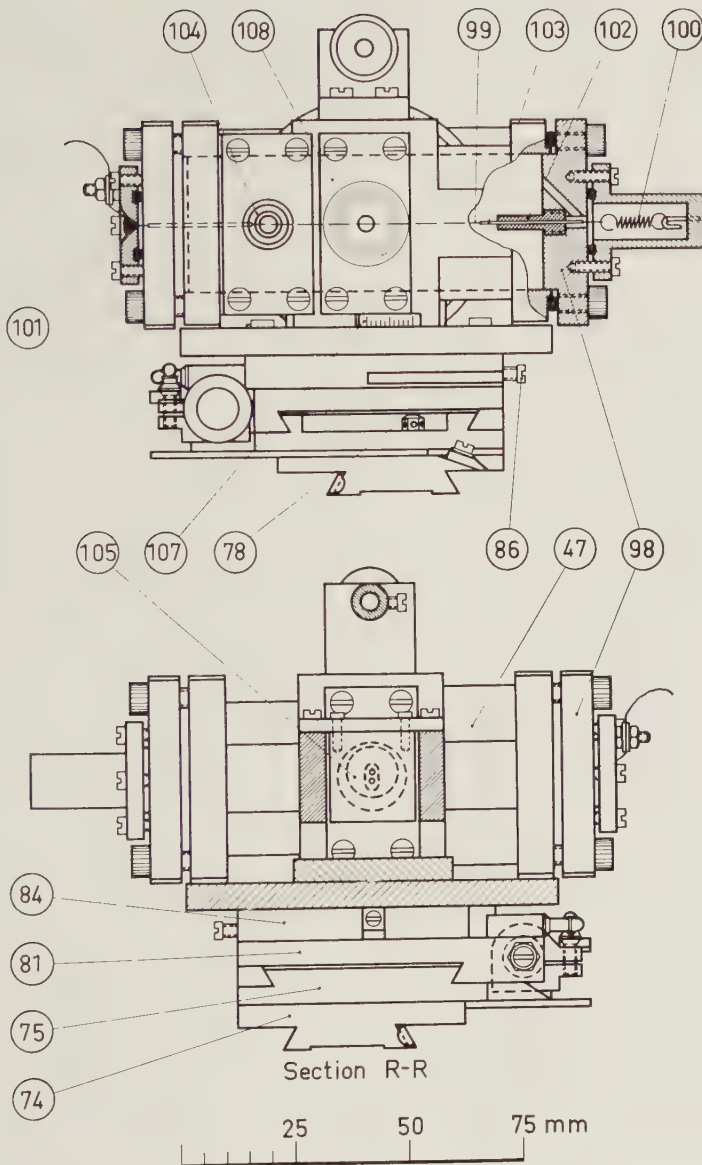


Fig. 52. View of the back of the registering system with the Geiger counter and section R—R in Fig. 51.

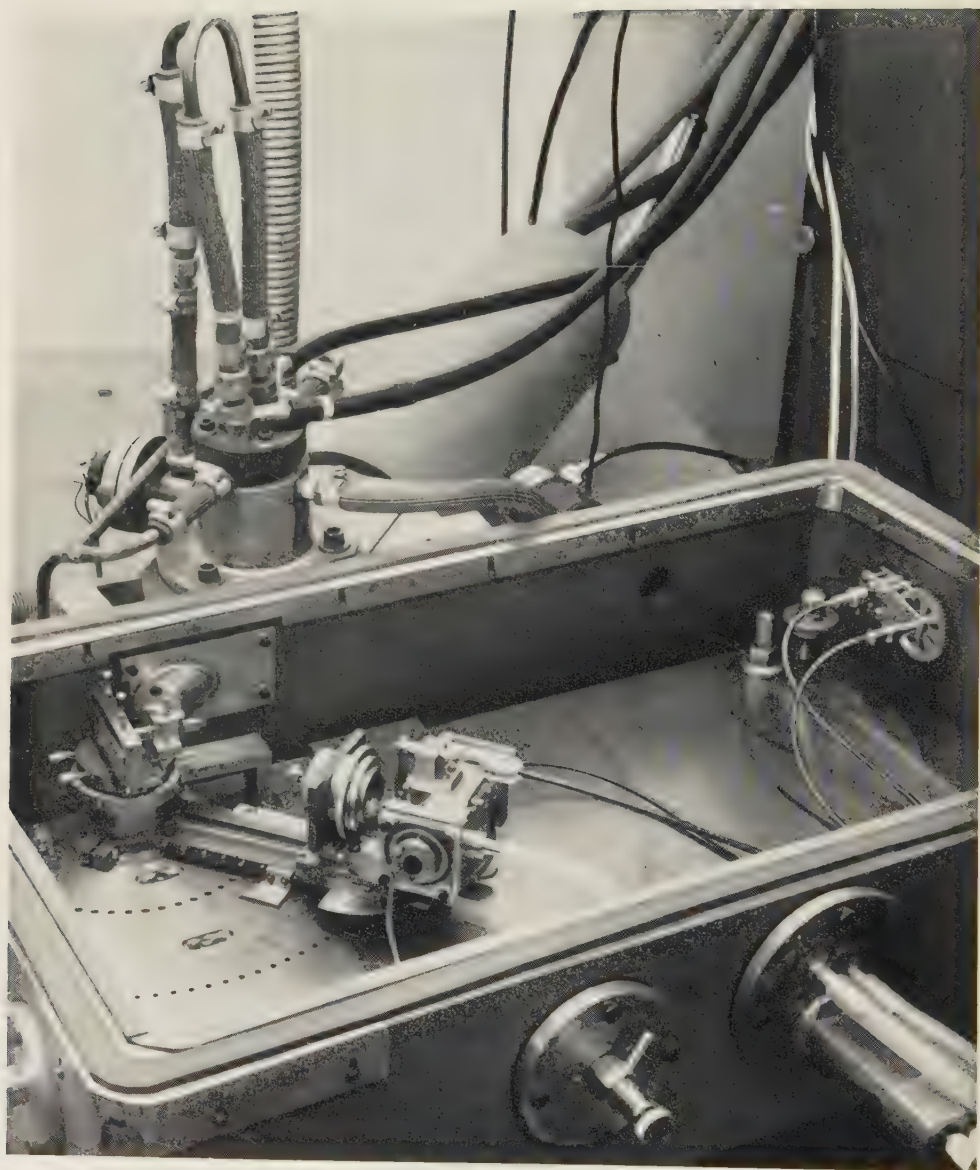


Fig. 53. Photograph of the spectrophotometer with the roentgen tube, the crystal table, and the Geiger counter.

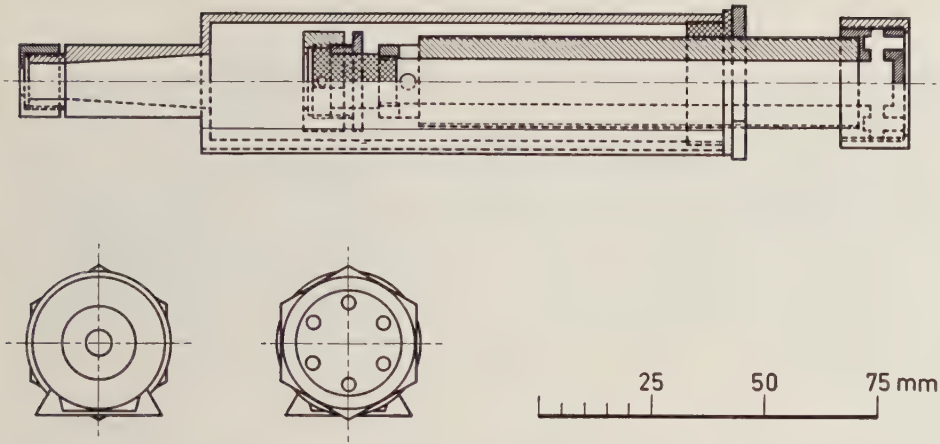


Fig. 54. Specimen holder for direct photographic registering utilizing continuous roentgen radiation.

holes on each side of the symmetric horizontal plane through the counter. The movable brass plate (106) serves to cover the holes alternately. This movement is transferred from (41) in Fig. 44. When total reflection is utilized simultaneously with the Geiger counter, the angle is adjusted by means of the nonius scale (107) on the circular scale on the plate (94).

A view of the spectrophotometer with the roentgen tube, the crystal table and the Geiger counter is shown in Fig. 53. The spectrometer can also be used to register continuous roentgen radiation. The specimen holder in Fig. 54 is then placed on the crystal table (45) after removal of the holder (38).

G. The Vacuum System

The vacuum system consists of a two stage rotary mechanical fore-pump (Pfeiffer No. 30100) and an oil diffusion pump (Distillation Products, type MC-500) (109), in Fig. 56, connected to the body of the spectrometer. The fore-pump is connected to a high vacuum valve (Fig. 55) by means of a tombak tube (110). A shunt tombak tube (111) connects the valve with the spectrometer, and the valve is also connected to the oil diffusion pump by (112). The bellow system (113) govern the movements of the metal plates (114). These plates tighten by means of rubber gaskets against turned surfaces on the central part of the body of the valve (115).

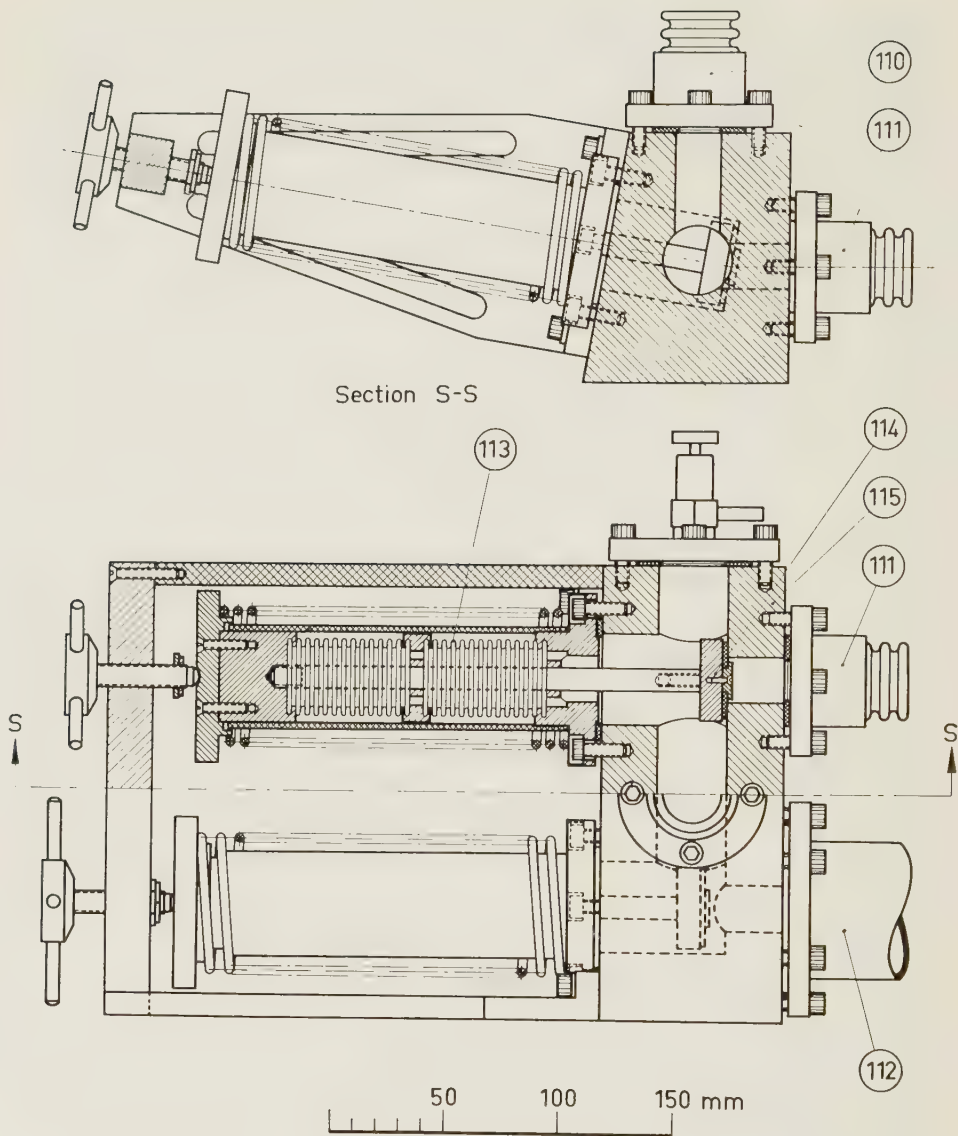


Fig. 55. The high vacuum valve.

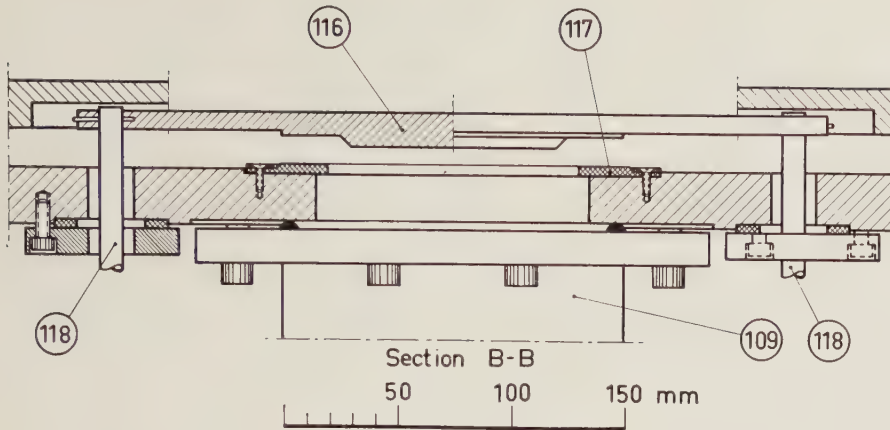


Fig. 56. Section B—B in Fig. 44 through the high vacuum valve between the spectrophotometer and the oil diffusion pump.

The body of the spectrometer can be shut off vacuum-tight from the oil diffusion pump by means of a steel plate (116) in Fig. 56 (Section B—B in Fig. 44). The rubber gasket (117) secures the vacuum-tightening. This is performed by vertical movements of the plate (116), transferred from a bellows system of the same principle construction as in the valve in Fig. 55, by means of the steel rods (118).

The vacuum is measured by means of a Philips Gauge, type PHG-09. In most of the experiments a vacuum of about 10^{-5} mm Hg was obtained.

H. The High Voltage Supply

The high voltage supply (wiring diagram Fig. 57) consists of two high tension transformers, one for a maximum of 20 kV and the other for 3.5 kV, common control system and B-battery eliminator. This comprises the air-cooled transformers T 1 and T 2, with connected tubes which furnish tension to the control system and current to the regulating transducers TD 1 and TD 2, which govern the high tension and the filament current to the roentgen tube, respectively.

By means of the change-over switches S 9 and S 10 one of the high tension transformers is alternately connected to the control system. By turning on the switch S 3 the rectifier tubes in the high tension transformer receive filament

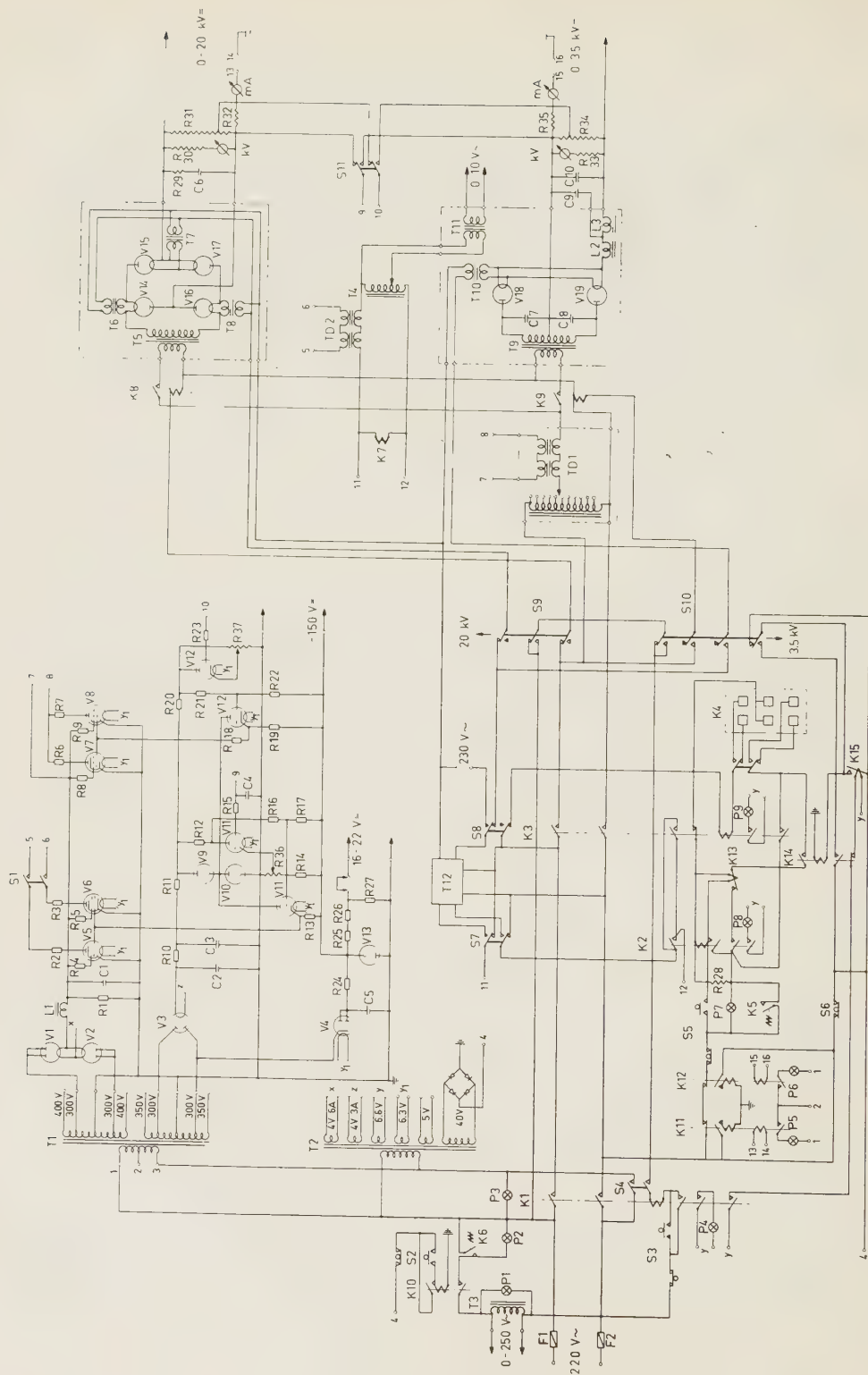


Fig. 57. Wiring diagram of the high voltage supply.

R1	20 k Ω	C1 4 μ F	T1	Voltage air-cooled transformer 110 and 220 V / 2 $\times$ 300 and 400 V—0.6 A	P1 Pilot light for oil diffusion pump
R2	50 Ω	C2 8 "	T2	Filament air-cooled transformer 230 V / 4 V—6 A, 4 V—3 A, 6.3 V—8 A, 6.6 V—4 A	P2 " " water relay K6
R3	50 "	C3 8 "		5 V—2 A, 0—10—40 V—0.6 A	P3 " " transformer T1
R4	200 "	C4 2 "			P4 " " contactor K1
R5	200 "	C5 4 "			P5 " " twocoil relay K11
R6	50 "	C6 0.5 "			P6 " " " K12
R7	50 "	C7 0.001 "			P7 " " water relay K5
R8	200 "	C8 0.001 "			P8 " " contactor K2
R9	200 "	C9 2 "			P9 " " K3
R10	3 k Ω	C10 3 "			
R11	1.3 "				
R12	600 "				
R13	100 "				
R14	10 "				
R15	100 "				
R16	2 M Ω				
R17	1.7 "				
R18	100 k Ω				
R19	100 "				
R20	600 "				
R21	2 M Ω				
R22	1.7 "				
R23	100 k Ω				
R24	5 "				
R25	10 "				
R27	3 "				
R28	200 "				
R29	100 Ω				
R30	30 M Ω				
R31	60 "				
R32	200 Ω				
R33	5 M Ω				
R34	10 "				
R35	200 Ω				
R26	Potentiometer 10 k Ω				
R36	" 10 "				
R37	" 50 "				
V1	AZ 50		TD1	Regulating transductor for high tension	S1 Switch for stabilization of current
V2	AZ 50		TD2	" " " current (roentgen tube)	S2 " " oil diffusion pump
V3	AZ 50				S3 " " filament
V4	EZ 40				S4 " " transformer T1
V5	EL 34				S5 " " high tension
V6	EL 34				S6 Breaker " twocoil relays K11 and K12
V7	EL 34				S7 Change-over switch for filament
V8	EL 34				S8 " " " stabilizing transformer
V9	VR 150				S9 " " " 3.5 and 20 kV
V10	VR 105				S10 " " " 3.5 and 20 kV
V11	6SL7				S11 " " " stabilization of current and high tension
V12	6SL7				
V13	VR 150				
V14	8020 RCA				F1 Fuse 25A
V15	8020 "				F2 " 25A
V16	8020 "				
V17	8020 "				
V18	872 A "				
V19	872 A "				
K1					
K2					
K3					
K4					
K5					
K6					
K7					
K8					
K9					
K10					
K11					
K12					
K13					
K14					
K15					

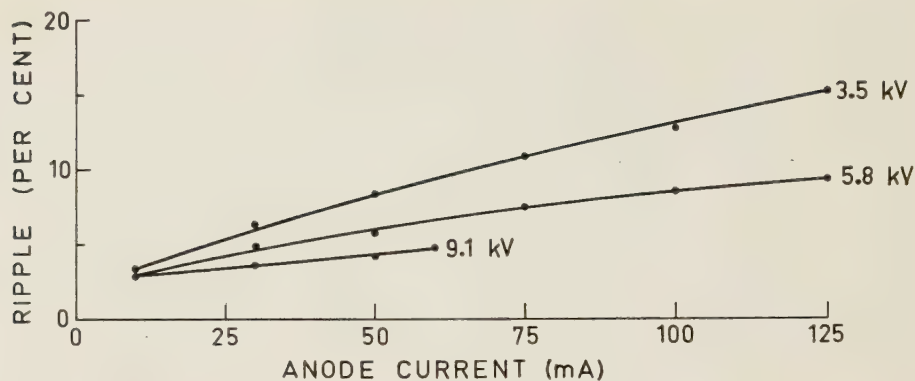


Fig. 58. The ripple of the high tension from the 20 kV transformer.

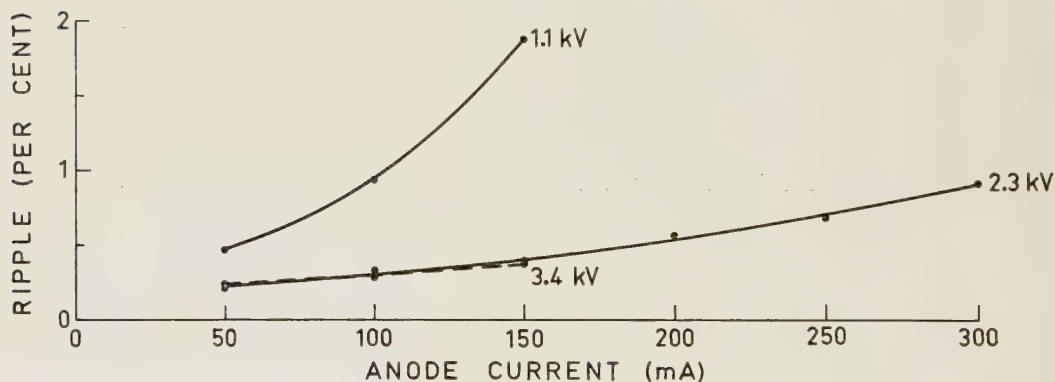


Fig. 59. The ripple of the high tension from the 3.5 kV transformer.

current. To secure sufficient temperature in the tubes, the high tension is, after turning on switch S 5, delayed by means of the time delay relays, K 13 for the transformer 20 kV and K 15 for the transformer 3.5 kV. The water relay K 5 controls the water cooling of the roentgen tube.

The high tension is regulated by means of the change-over switch in the transducer TD 1 and the potentiometer R 37. The automatic control of the regulated high tension is obtained by feeding back a certain fraction of the tension over the change-over switch to the tube V 12. In this way the current from the tubes V 7 and V 8, passing through the transducer TD 1, is changed, leading to a changed tension in the transformers T 5 and T 9 alternately. If the maximum current of the high tension transformers is

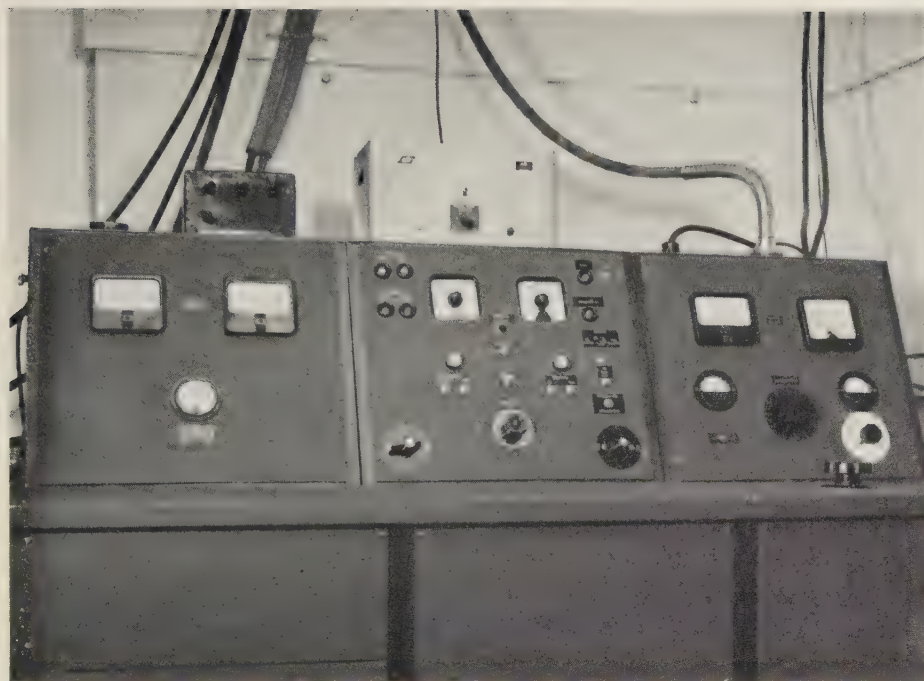


Fig. 60. Photograph of the instrument panel of the high voltage supply.

exceeded, the high tension is automatically turned off by the max. current twocoil relays K 11 and K 12, respectively. These are reset by means of the breaker S 6.

The anode current in the roentgen tube, connected to the high voltage supply, is regulated by means of the filament current to the roentgen tube and is controlled by means of the adjustable transformer T 4 and the potentiometer R 36.

The automatic adjustment of the anode current is obtained by feeding back the potential drop over the fixed resistances R 32 and R 35. The potential drop from these resistances, respectively, reaches the tube V 11 through the change-over switch S 11. The current from the tubes V 5 and V 6 through the transducer TD 2 acts upon the tension to the adjustable transformer T 4.

The solenoid valve automatically shuts off the cooling water to the roentgen tube when the tension is turned off. The water relay K 6 controls the cooling water to the oil diffusion pump.

During the experiments C 6 has been $1\ \mu\text{F}$, C 9 $4\ \mu\text{F}$ and C 10 $6\ \mu\text{F}$ in order to obtain a still smaller ripple. Fig. 58 shows the ripple as a function of the anode current of the roentgen tube at three different voltages for the 20 kV transformer. Corresponding diagrams for the 3.5 kV transformer are shown in Fig. 59.

A view of the control system for the high voltage supply is shown in Fig. 60.

CHAPTER 13

The Experimental Procedures

A. The Adjustment of the Spectrophotometer

The adjustment of the path of the roentgen beam in the spectrophotometer is performed with the starting-point at the registering system. The centre of the slit (87) in Fig. 50 is situated at a fixed distance from the bottom plate (49). Therefore, the centre of the opening of the crystal must be adjusted relative to the slit. When this has been performed, the bottom plate (49) is adjusted relative to the focal spot of the roentgen tube.

The experimental arrangement for the adjustment is schematically shown in Fig. 61. A second slit S_4 with an attached light source L is placed behind the slit (87), denoted S_3 . The axes of rotation of S_3 and S_4 coincide and are parallel with the bottom plate (49).

1) The edges of the slit S_4 are adjusted until the plane through the openings of the slits in the horizontal position is parallel with the bottom plate.

2) The cylindrical diffracting surface of the crystal C is adjusted at right angles to the bottom plate by the three screws (60) (Fig. 45, Fig. 47). The right position is obtained when the image of the horizontal opening of the slit S_3 , reflected by the crystal, coincides with the opening of the slit.

3) The centre of the diffracting crystal surface is adjusted to coincide with the axis of rotation of the crystal table (45) by means of the four screws (58) (Fig. 45). The right position is controlled by means of a centering microscope.

4) The rotation of the crystal is read off on the adjustable nonius V_1 in Fig. 61, i.e. the nonius on the arm (68) in Fig. 45. The corresponding nonius V_2 on the arm (46) and (70), respectively, is fixed. A difference of 90° between the position of the arm and the crystal table (45) is obtained, when the image of the vertical opening of the slit S_3 , reflected by the crystal, coincides with the opening of the slit. The nonius V_1 is adjusted until the right angular difference between the position of the crystal and the registering system is read off.

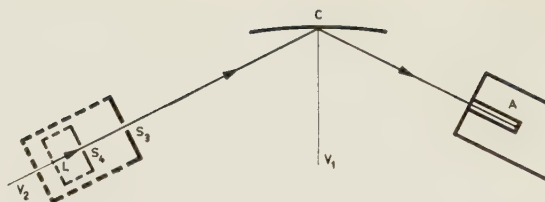


Fig. 61. Schematic illustration of the experimental arrangement for the adjustment of the spectrophotometer.

5) Finally, the path of the roentgen beam must be adjusted relative to the bottom plate (49) with the adjusted crystal and the registering system. The cathode is then removed from the roentgen tube and V_1 is fixed at 45° and V_2 at 90° . The bottom plate (49) is adjusted by means of the four screws (50) (Fig. 44) until the image of the horizontal slit S_3 , reflected by the crystal, falls in the centre of the focal spot A. The bottom plate (49) is then adjusted in the horizontal direction until the image of the vertical opening of the slit S_3 falls in the centre of the focal spot.

6) Final small adjustments are performed, when the images of different roentgen emission lines are registered on photographic plates, placed on the Rowland circle.

B. The Selection of Suitable Monochromatic Radiation for Elementary Analyses

The monochromatic radiation utilized for elementary analyses can be obtained either from a continuous roentgen spectrum or from characteristic roentgen emission spectra. In the present investigation the continuous spectrum could be utilized only at wavelengths shorter than about 3 kX.U., when fine-grained photographic emulsions were utilized to register the absorption image of the specimen. Considerably higher intensities of monochromatic roentgen radiation can be obtained, when utilizing suitable roentgen emission lines of different elements. These spectra have been briefly discussed in Chapter 1: B. As can be seen in the lower diagram in Fig. 3, a few emission lines for each element have high relative intensities, and only such lines should be utilized.

The intensity of a roentgen emission line increases with the voltage across the roentgen tube (compare equation (18)). However, the voltage must be kept below a certain value, if monochromatic roentgen radiation shall be

obtained by diffraction by crystals, as discussed in Chapter 3:B. The roentgen ray spectrophotometer, described in the previous chapter, was designed only for primary excitation of the characteristic roentgen emission lines.

It is important to select roentgen emission lines with wavelengths as close as possible to the wavelength of the absorption edge of the element to be investigated [29]. The systematic error of the elementary analyses will then be rather small as discussed in Chapter 11:B.

C. The Influence of Broadening Effects Inherent in the Bent Crystal Technique

The diffracted monochromatic roentgen beam is not exactly focused, when utilizing the bent crystal technique according to Johann, which was discussed in Chapter 3:C. The magnitude of the broadening b , tending in the direction of shorter wavelengths, is given by equation (42). The vertical extent h_1 of the focal spot has also a broadening effect b_1 , though *tending in the direction of longer wavelengths*. The following expression has been deduced [85]:

$$b_1 = \frac{h_1^2}{8 (R \sin \varphi + a_1) \cos \varphi} \dots\dots\dots (80).$$

R is the radius of curvature of the crystal, φ the glancing angle, and a_1 the distance between the focal spot and the crystal.

In the spectrophotometer described, $a_1 = 195$ mm, $h_1 = 6.5$ mm (see Chapter 7:A), $R = 200$ mm or 385 mm, and φ from 10° to 70° . The highest value of b_1 will then be about 0.04 mm. Therefore, the broadening b_1 can be completely neglected, when performing elementary analyses on a cytochemical scale.

The effect of the broadening b is greater, especially at small glancing angles. As compared with the intensity of a strong roentgen emission line, the intensity of the roentgen radiation in the continuous spectrum at adjacent wavelengths is small. Therefore, the influence of the broadening b is negligible when roentgen emission lines are utilized at the elementary analyses. When a continuous roentgen spectrum is utilized, however, absorption measurements, performed at wavelengths immediately adjacent to the wavelength of the absorption edge, may be affected with a systematic error.

The analyses of calcium were performed, utilizing continuous roentgen radiation. For these analyses, the linear opening of the crystal (quartz,



Fig. 62. Photo-micrograph, $\times 4.2$, of the Cr $K\alpha_{1,a_2}$ line, registered on an inclined plate.

$d = 3.336$ kX.U.) was 10 mm, whereas the whole opening (20 mm) was utilized at most of the other analyses. In this case the broadening b is reduced by a factor of 4, and has no influence upon the elementary analyses. As the photographic emulsions were placed at right angles to the roentgen beam and not along the tangent to the Rowland circle, no additional broadening occurred.

The radius of curvature of the bent crystals were determined by registering a roentgen emission line on a photographic plate, forming an angle of 30° relative to the bottom plate of the spectrometer. The sharply defined portion of the image of the emission line is situated on the Rowland circle. The distance between this part of the image and the centre of the opening of the crystal, divided by $\sin \varphi$, is the radius of curvature of the crystal. Fig. 62 shows the Cr $K\alpha_{1,a_2}$ line, registered in the second order ($d = 3.336$ kX.U., $R = 385$ mm) on such an inclined plate.

D. Registering with the Geiger Counter

The Geiger counter has been used in some model experiments utilizing extremely soft roentgen radiation. A gas mixture, consisting of 70 per cent argon and 30 per cent methylal, was utilized at a pressure of 65 mm Hg. The plateau of the counter extended from 1000 to 1250 volts, and the relative increase of the number of counts per 100 volts was 3 per cent. A SC-34 Precision Ratemeter (Tracerlab) delivered a well stabilized high voltage to the counter and registered the integrated number of counts during 2.5, 10, 40, or 120 seconds, alternately. Fig. 63 shows the high vacuum roentgen ray spectrophotometer, the Precision Ratemeter and the apparatus utilized to obtain the gas mixture for the Geiger counter.

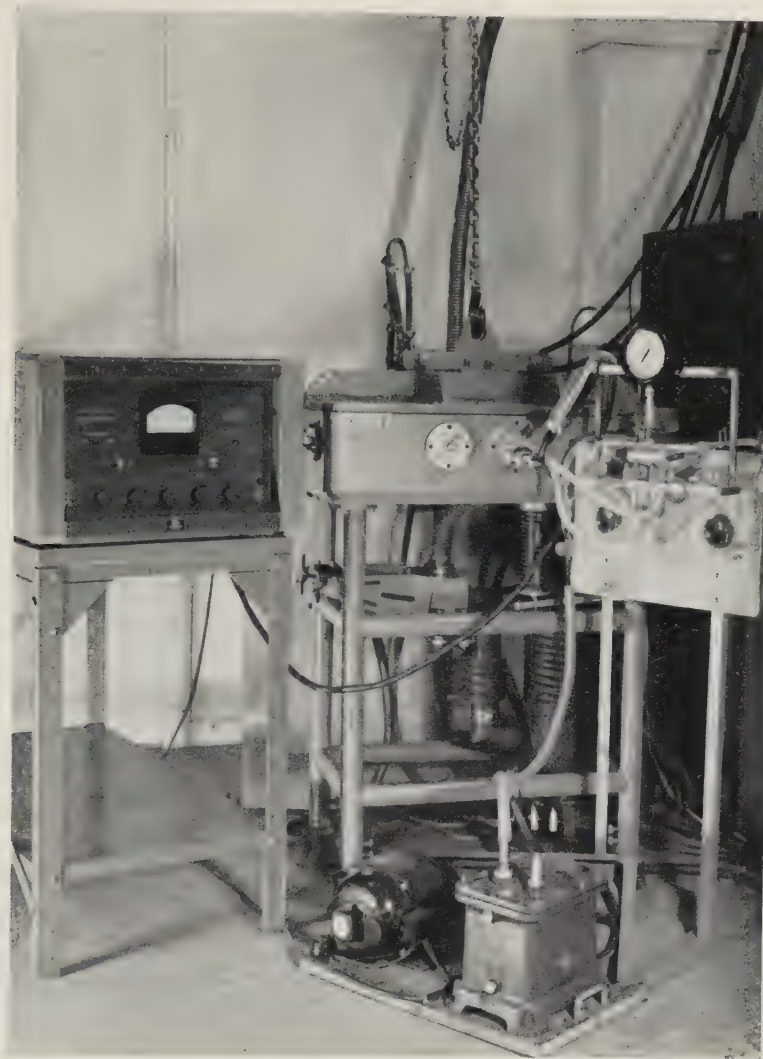


Fig. 63. View of the high vacuum roentgen ray spectrophotometer, the Precision Ratemeter, and the arrangements for filling the Geiger counter.

In a model experiment, performed to investigate the K absorption edge of oxygen, one of the holes (0.35 mm in diameter) in the holder (105) in Fig. 52 was covered with a Parlodion foil with a known weight per unit area. Monochromatic roentgen radiation was obtained from the continuous roentgen spectrum, by diffraction by a Ba-Cu-stearate crystal. By means of

the micrometer screw the Geiger counter was displaced at right angles to the roentgen beam. The plane of the Parlodian foil cut the Rowland circle at the point, corresponding to the K absorption edge of oxygen at 23.35 Å.U. At different positions of the counter, the roentgen radiation transmitted through the Parlodian foil and the radiation passing through the uncovered hole were registered, thus making it possible to correct for an uneven distribution of the intensity of the roentgen radiation. The numbers of counts at the position of the absorption edge, were extrapolated, and the amount of oxygen was calculated according to equation (79), and agreed with the correct value within about ± 10 per cent. The values were, however, unreliable, as the difference between the mass absorption coefficients is not accurately known, and the effect of a fine structure must also be considered.

E. The Photographic-Photometric Procedure

The curve, describing the density of a photographic emulsion as a function of the exposure to roentgen radiation, has generally an initial straight portion, which was discussed in Chapter 4:A. This can be utilized to determine the ratio between roentgen intensities, provided the corresponding densities are situated within the straight portion of the curve. However, the extent of the initial straight portion varies for different photographic emulsions and is influenced by the wavelength of the roentgen radiation.

In the present investigation these curves were determined according to the principle, schematically illustrated in Fig. 64. A roentgen emission line, diffracted by the bent crystal, was focused in the opening of the slit S_1 on the Rowland circle. At a distance behind the slit S_1 a rotating sector S_2 was placed immediately in front of a photographic emulsion F. This arrangement was utilized, as the distribution of the intensity of the monochromatic roentgen radiation is much more homogeneous at the position of F than at S_1 , thus increasing the accuracy of the photometric determinations of the densities.

A view of the experimental arrangement is shown in Fig. 65, where the sector, mounted together with a motor and the slit S_1 , has been separated from the film. Stepped series of exposures were obtained on the same photographic emulsion, as shown in Fig. 66, as the photographic plate could be displaced in a plane at right angles to the roentgen beam. The sector was cut to give a ratio of $\sqrt{2}$ between successive exposures.

Fig. 64. Position of the rotating sector in the roentgen beam.

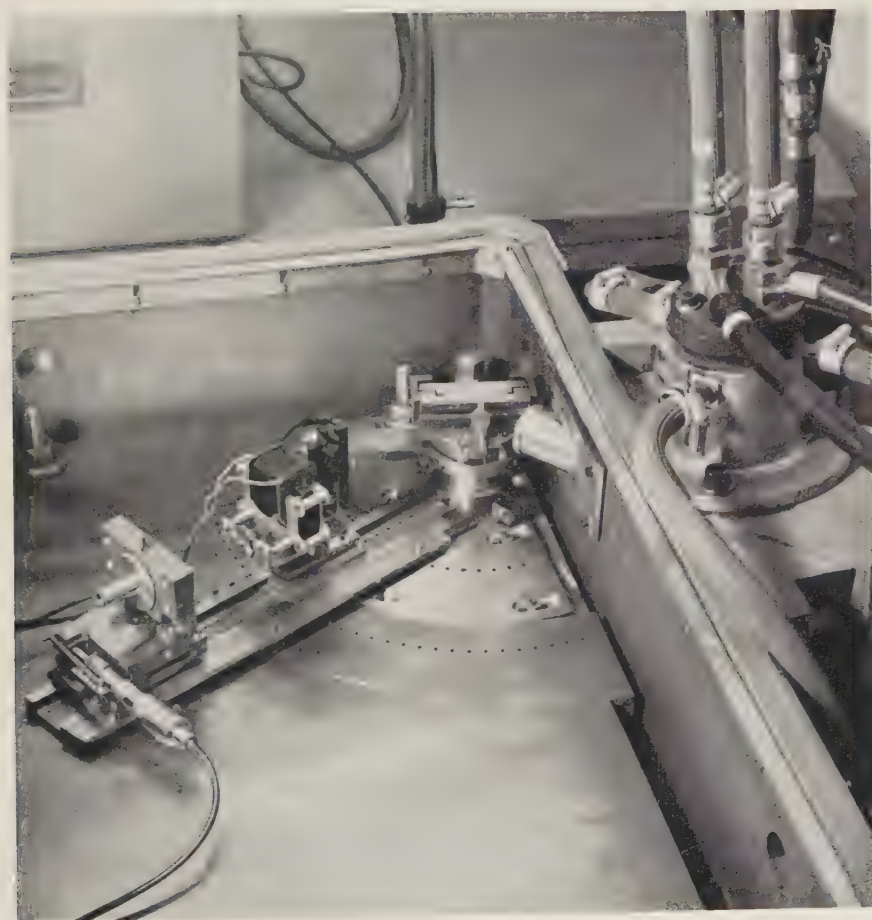
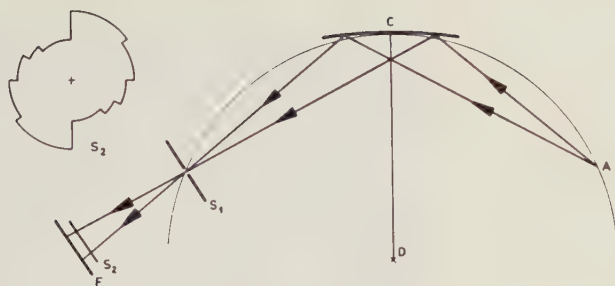


Fig. 65. View of the spectrophotometer with the sector inserted between the crystal table and the registering system.

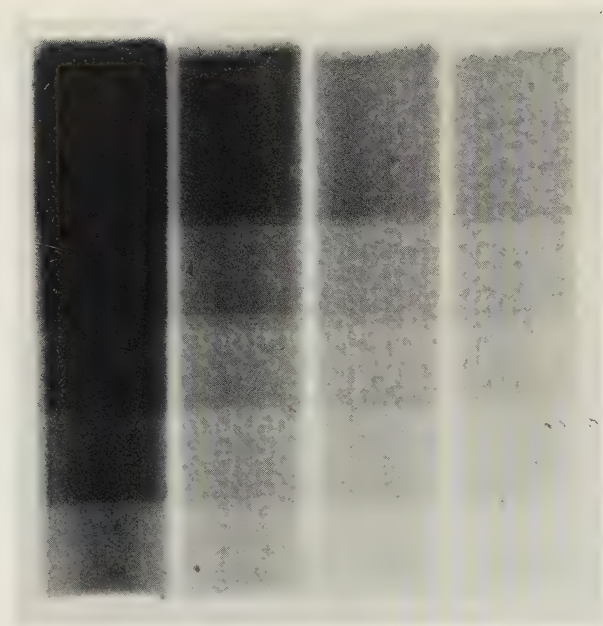


Fig. 66. Photo-micrograph, $\times 10$, of a stepped series of exposures on a Kodak High Resolution plate.
Radiation: Ti $K\alpha_{1,2}$ ($\lambda \approx 2.74$ kX.U.).

Kodak High Resolution plates were exposed to monochromatic roentgen radiation, Ti $K\alpha_{1,2}$ and Nb $L\alpha_{1,2}$, and developed, as described in Chapter 8: E. The densities were determined by means of the photometric procedure, described in Chapter 8: D.

The densities corresponding to a stepped series of exposures were plotted as a function of the logarithm of the exposure. In order to obtain a common density curve the individual curves were displaced along the axis of abscissae, until a smooth curve was obtained. From the displacement of the individual density curves a common exposure scale was calculated. Accordingly, the densities could then be plotted as a function of the roentgen intensity, as shown in Fig. 67 and Fig. 68. It can be seen that *the density of this photographic emulsion is a linear function of the intensity of the roentgen radiation, incident upon the emulsion, up to a density of about 0.6 at 2.74 kX.U., and 0.5 at 5.71 kX.U.*

In the roentgen absorption images of the specimen, registered at wavelengths on both sides of the absorption edge of the element to be investigated,

a density of 0.5 was not exceeded. In all other respects the photographic-photometric procedure at the elementary analyses was identical with the one utilized at the total dry weight determinations, described in Chapter 8.

In order to obtain an even exposure of the microradiograms, when roentgen emission lines were utilized, the same general arrangement was used, as shown in Fig. 64. The only difference was that the specimen, mounted on a metal disc as described in Chapter 8: B, was substituted for the sector S_2 and in close contact with the fine-grained photographic plate F. The slit on the metal disc was parallel with the opening of the slit S_1 on the Rowland circle, (87) in Fig. 50. The opening of the slit S_1 was generally between 0.7 and 1.0 mm.

The arrangement for *total reflection*, (95) in Fig. 50, was tested with chromium and gold as reflecting surfaces. The roentgen tube was run at a voltage high enough to generate roentgen radiation with wavelengths, diffracted by the bent crystal in the second and higher orders. The incident glancing angle at the total reflection was calculated according to equation (39 a). The angle corresponded to the angle of critical grazing incidence for roentgen radiation with a wavelength of $3\lambda/4$, where λ was the wavelength of the roentgen radiation, diffracted in the first order. Then roentgen radiation, diffracted in the second and higher orders, was cut out. When the roentgen beam had been totally reflected, a higher intensity of monochromatic roentgen radiation with the wavelength λ could be obtained, as compared with the intensity of the same monochromatic roentgen radiation generated at a voltage, where diffraction in the second order was not possible. However, the distribution of the intensity in the totally reflected beam was inhomogeneous, precluding the utilization of combined diffraction and total reflection at the elementary analyses of biologic objects.

The photographic-photometric procedure must be used *at quantitative elementary analyses on a cellular level*. The Geiger counter cannot be utilized, as an aperture with a diameter of about 5μ is extremely difficult to make. This has been discussed in the introduction of Chapter 4.

The wavelength region to be utilized at the photographic-photometric procedure is restricted by the low intensities of monochromatic roentgen radiation at long wavelengths. The intensity of monochromatic roentgen radiation, diffracted by Ba-Cu-stearate crystals, was too low to be registered on fine-grained photographic emulsions. Concave gratings (see Chapter 3: D) could not be used in the spectrophotometer which was constructed especially

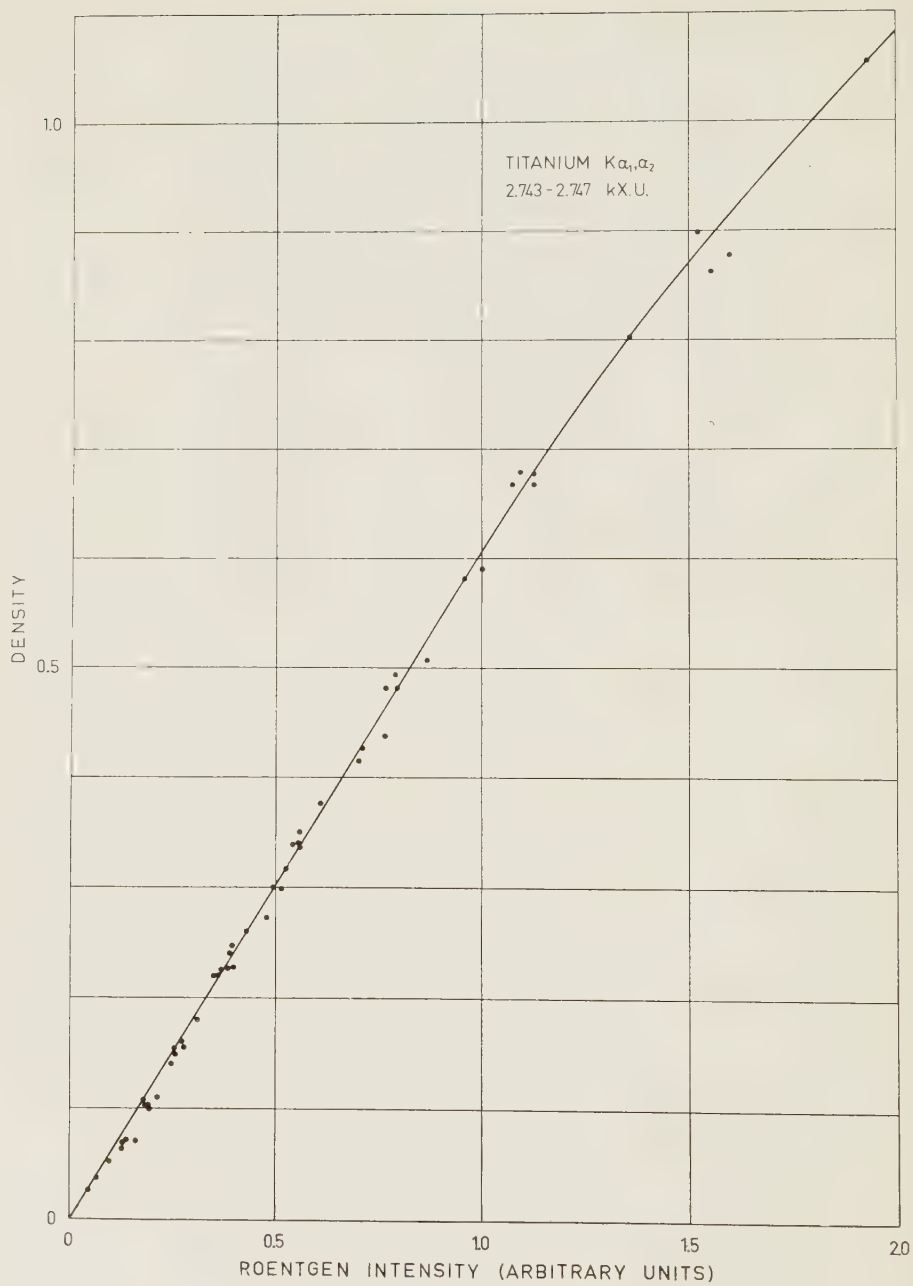


Fig. 67. The density as a function of the roentgen intensity for Kodak High Resolution plates.

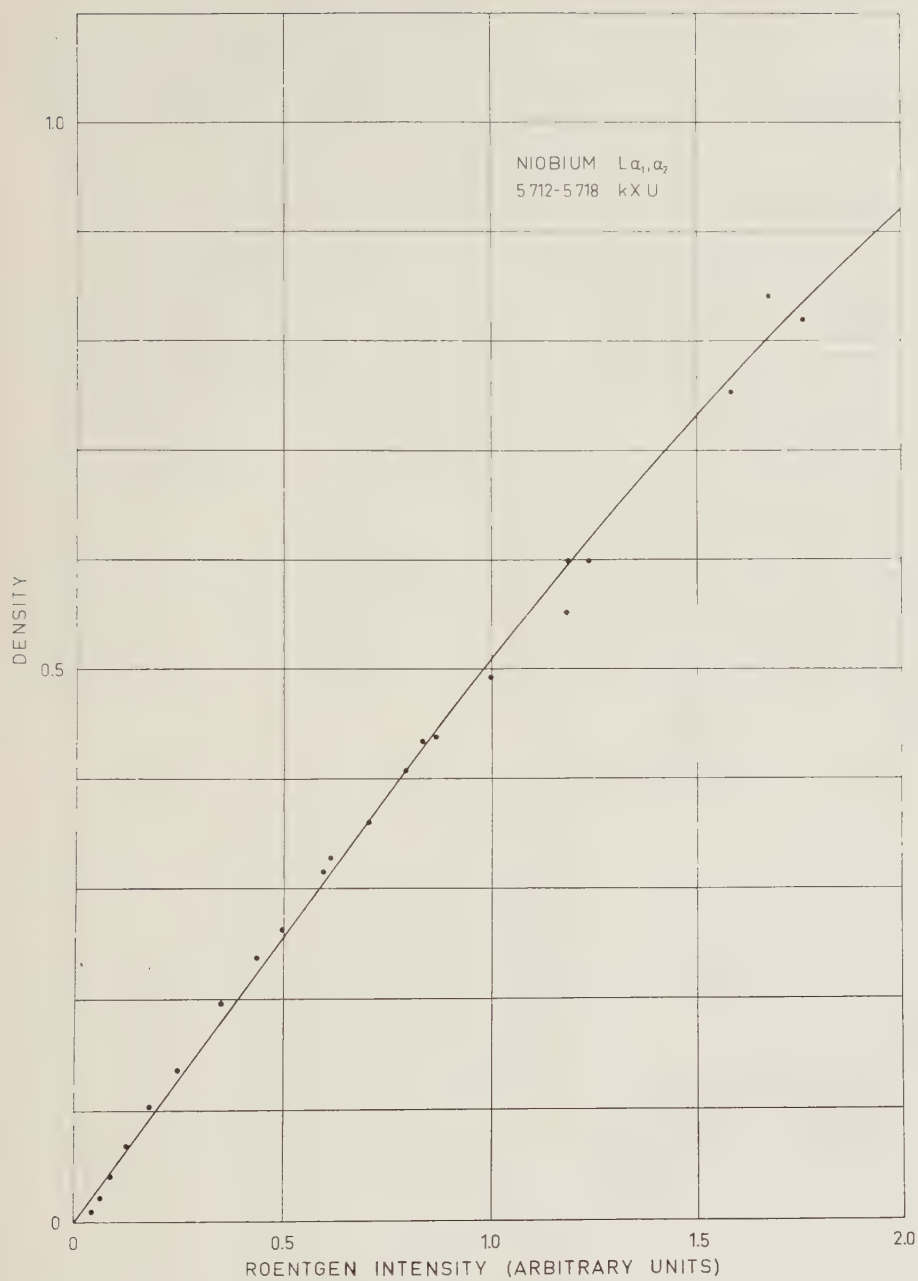


Fig. 68. The density as a function of the roentgen intensity for Kodak High Resolution plates.

for bent crystals. Further experiments are necessary to settle the most advantageous experimental arrangement to obtain monochromatic roentgen radiation with high intensities at wavelengths longer than about 19 kX.U. At wavelengths shorter than this limit the method utilizing bent crystal is superior to other methods.

F. The Error of the Photographic-Photometric Procedure

In equations (78) and (79) the different intensities of roentgen radiation are measured experimentally by the photographic-photometric procedure. As the densities did not exceed 0.5, the following expression is obtained according to equation (44):

$$k_2 I = D = \log \frac{i_0}{i_1} = 0.4343 (\ln i_0 - \ln i_1) \quad \dots\dots\dots (81),$$

where k_2 is a constant of proportionality. As only ratios between roentgen intensities occur in equations (78) and (79), the roentgen intensity I can be measured in arbitrary units. Hence the unit of D is selected, and consequently $k_2 = 1$. The standard error dI of I is then calculated according to equation (67):

$$dI = \pm 0.4343 \sqrt{\left(\frac{di_0}{i_0}\right)^2 + \left(\frac{di_1}{i_1}\right)^2} \quad \dots\dots\dots (82),$$

where di_0 and di_1 are the standard errors of the photo-currents i_0 and i_1 , respectively. In the present work i_0 is about 1 μA , and as the density has not exceeded a value of 0.5, the minimum value of i_1 has been about 0.3 μA . As the same photographic-photometric procedure was utilized as for total dry weight determinations, $di_0 \approx di_1 \approx 0.01 \mu\text{A}$ according to Table XIV. However, these values were obtained, when the photometric procedure was performed on areas with a size of 1.5 μ^2 . At the photometric procedure for the elementary analyses an area of about 7 μ^2 was measured. Therefore, di_0/i_0 and di_1/i_1 can be assumed to have an average value of about 0.01. The average value of dI is then about ± 0.006 .

At the photometric procedure 10 measurements were performed on each structure, and the standard error of the mean of the density was calculated. An average value of about ± 0.002 was obtained, in good agreement with the theoretical calculations.

The effect of an inhomogeneous distribution of the absorbing material within the biologic object may influence upon absorption measurements, utilizing roentgen radiation. ENGSTRÖM and WEISSBLUTH [195] have calculated correction formulae, applicable to model substances consisting of small, non-overlapping spheres or cubes of equal size.

The elementary analyses to be described have been performed on specimens with a thickness of about $10\ \mu$. For the measured areas within the absorption images of these specimens the effect of inhomogeneity is smaller than the influence of the granularity of the photographic emulsion. When total dry weight determinations were performed on thin sections of biologic objects the area measured at the photometric procedure was about $1.5\ \mu^2$. For the measured, small areas the error, introduced by the granularity of the photographic emulsions, was much more important than a possible, inhomogeneous distribution of the absorbing material in the specimens under investigation.

Applications of Elementary Analyses on a Cellular Level

When applying the quantitative elementary analyses to thin biologic specimens, it was possible to determine the amounts of calcium, phosphorus, and sulphur on a cytochemical scale. The K absorption edges of these elements are situated in a wavelength region suitable for the bent crystal technique. The photographic-photometric technique, described in the preceding chapter, has been used, and all the microradiograms have been registered on Kodak High Resolution plates. The photometric determinations of the densities in the enlarged images of the microradiograms were performed on areas with a size of about $7 \mu^2$, 10 photometric measurements on each analysed structure.

One of the main advantages of the bent crystal technique is the possibility to obtain a homogeneously distributed intensity of monochromatic roentgen radiation, when the specimen is placed at a distance behind the slit, situated on the Rowland circle. On the other hand, the geometric unsharpness was about 1μ for sections with a thickness of between 10 and 15μ , when the whole opening of the bent crystal was utilized. The spatial resolving power was therefore determined by the geometric unsharpness and not by the granularity of the photographic emulsion.

The time of exposure has varied between 30 minutes and 4 hours in the following applications.

A. Determination of Calcium and Phosphorus in Osseous Tissue

Calcium and phosphorus form the principal inorganic constituents of bone. Chemical analyses have revealed considerable variations in the quantitative distribution of these elements in osseous tissue in different functional and pathologic conditions. Microradiographic studies *in situ* of compact longitudinal bone have shown that the different Haversian systems generally show a varying absorption of continuous roentgen radiation, generated at about 5 or 6 kV [109]. Quantitative elementary analyses of the amounts of calcium and phosphorus in human compact bone were performed in the present investigation.

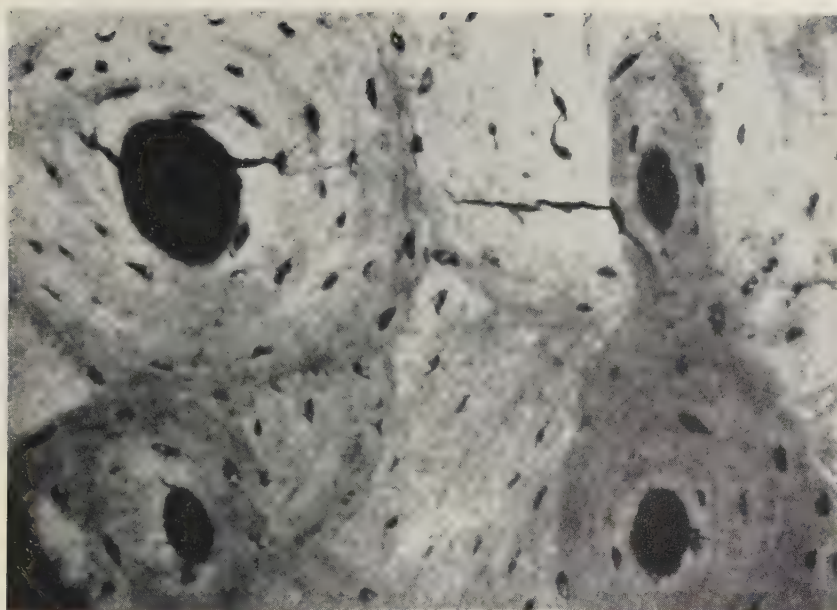
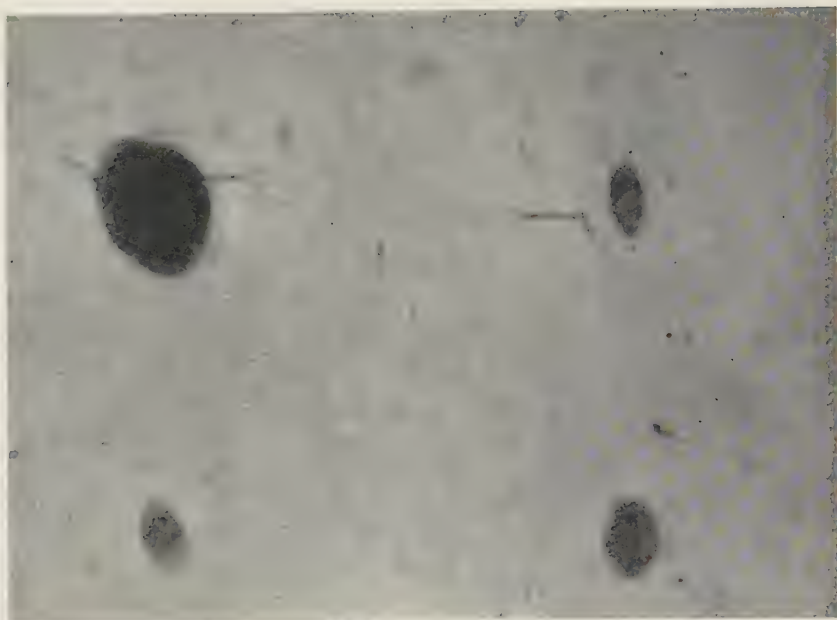


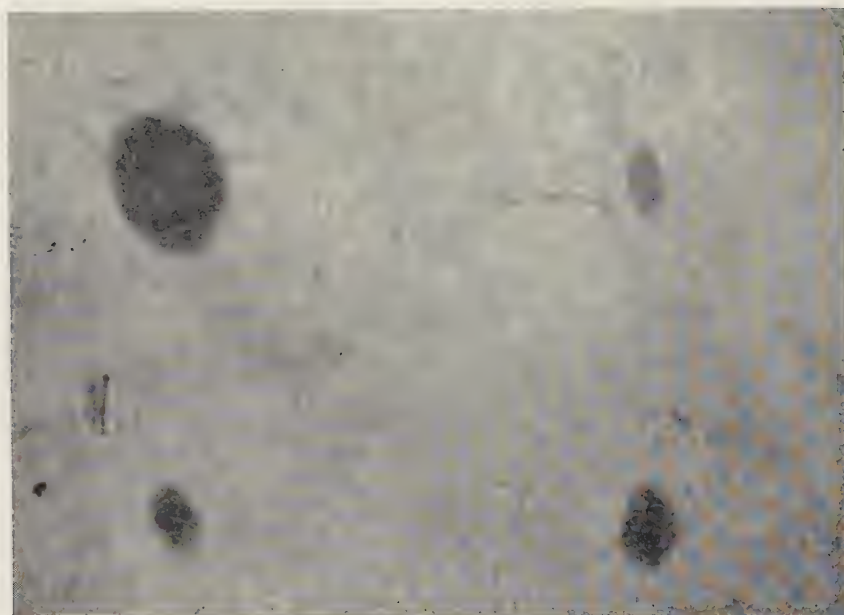
Fig. 69. Microradiogram, $\times 265$, of a $\sim 10 \mu$ thick ground slice of human compact bone, registered at 3.5 kV (continuous radiation).

Thin slices of fresh human compact bone (humerus from a boy, 11 years of age) were ground to a thickness of about 10μ and exposed to soft, continuous roentgen radiation, generated at 3.5 kV. In the microradiogram (Fig. 69) a considerable variation of the density, corresponding to different Haversian systems, can be seen. The same specimens were used to determine the amounts of calcium and phosphorus.

For the analysis of calcium no suitable roentgen emission lines are available. In the spectrophotometer designed, however, the intensity in the continuous roentgen spectrum from a tungsten anode, generated at a voltage of 7.0 kV, was high. Therefore, sufficient intensities of monochromatic roentgen radiation with wavelengths on both sides of the K absorption edge of calcium ($\lambda = 3.064 \text{ kX.U.}$) [13] could be obtained by diffraction by the bent crystal. A quartz crystal with a grating space of 3.336 kX.U. and a radius of curvature of 384 mm was utilized. The absorption images of the specimens were registered at $\sim 3.03 \text{ kX.U.}$ and $\sim 3.10 \text{ kX.U.}$, respectively. The absorption images of the same Haversian systems as in Fig. 69, registered at these wavelengths on each side of the K absorption edge of calcium, are shown in Fig. 70 a och b.



a



b

Fig. 70. Microradiogram, $\times 265$, of the same ground slice as in Fig. 69.
a) Registered at ~ 3.03 kX.U. b) Registered at ~ 3.10 kX.U.

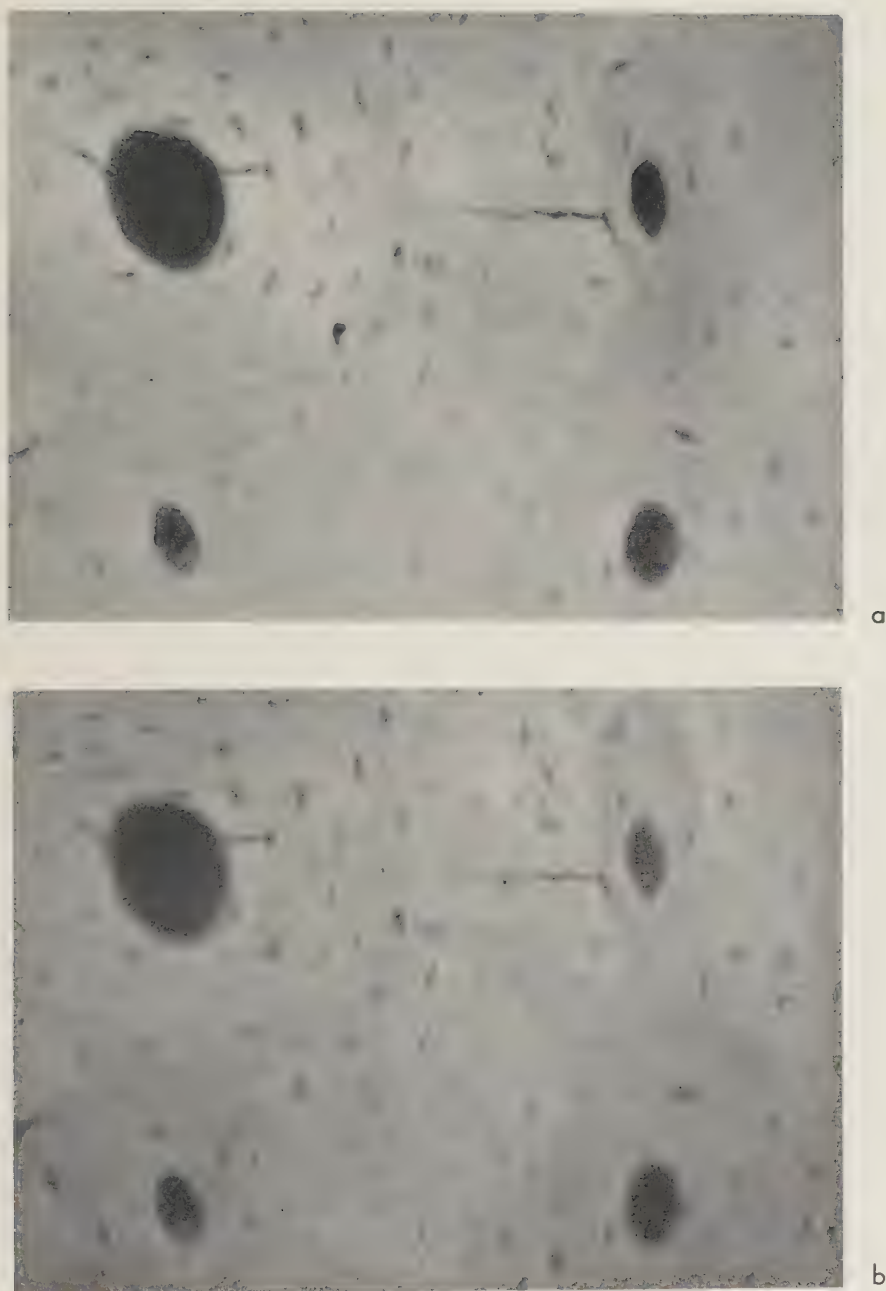


Fig. 71. Microradiogram, $\times 265$, of the same ground slice as in Fig. 69.
a) Registered at 5.71 kX.U. b) Registered at 5.82 kX.U.

Table XVII. Determination of the amount of calcium and phosphorus in two Haversian systems in human bone

Calcium						Phosphorus					
3.03 kX.U.			3.10 kX.U.			Nb $L\alpha_1 = 5.712$ kX.U.			Zr $L\beta_1 = 5.824$ kX.U.		
I_{01}	I_{11}		I_{02}	I_{12}		I_{01}	I_{11}		I_{02}	I_{12}	
	H 1	H 2		H 1	H 2		H 1	H 2		H 1	H 2
0.468	0.256	0.263	0.351	0.280	0.280	0.305	0.073	0.086	0.403	0.170	0.190
0.475	0.244	0.259	0.356	0.275	0.284	0.310	0.065	0.086	0.414	0.164	0.187
0.462	0.256	0.248	0.346	0.288	0.284	0.305	0.070	0.094	0.408	0.174	0.197
0.462	0.244	0.267	0.351	0.280	0.280	0.301	0.073	0.089	0.408	0.177	0.190
0.475	0.236	0.267	0.356	0.280	0.284	0.301	0.068	0.092	0.403	0.174	0.193
0.475	0.252	0.252	0.351	0.280	0.280	0.305	0.068	0.094	0.414	0.167	0.197
0.468	0.252	0.256	0.356	0.292	0.275	0.310	0.065	0.086	0.408	0.174	0.187
0.462	0.256	0.267	0.346	0.288	0.280	0.305	0.070	0.089	0.403	0.174	0.187
0.475	0.259	0.259	0.351	0.284	0.272	0.301	0.070	0.094	0.403	0.170	0.197
0.468	0.248	0.256	0.351	0.292	0.284	0.305	0.073	0.089	0.414	0.164	0.193
0.469	0.250	0.259	0.352	0.284	0.280	0.305	0.070	0.090	0.408	0.171	0.192
± 0.002	± 0.002	± 0.002	± 0.001	± 0.002	± 0.001	± 0.001	± 0.001	± 0.001	± 0.002	± 0.001	± 0.001
H 1 $m_{Ca} = (4.61 \pm 0.14) \mu\mu g \cdot \mu^{-2}$						H 1 $m_P = (2.76 \pm 0.07) \mu\mu g \cdot \mu^{-2}$					
H 2 $m_{Ca} = (4.06 \pm 0.12) \mu\mu g \cdot \mu^{-2}$						H 2 $m_P = (2.15 \pm 0.06) \mu\mu g \cdot \mu^{-2}$					

Photometric determinations of the densities, obtained in 10 areas within each of the enlarged images of two different Haversian systems, are presented in Table XVII. Applying equation (78) the amounts of calcium per unit area (μ^{-2}) were calculated. The correction factor in the numerator could be neglected, and the denominator had a value of 900.

For the analyses of phosphorus the characteristic roentgen emission lines Nb $L\alpha_1$ ($\lambda = 5.712$ kX.U.) and Zr $L\beta_1$ ($\lambda = 5.824$ kX.U.) were utilized. The K absorption edge of phosphorus is situated at 5.775 kX.U. [13.] The diffracting quartz crystal had a grating space of 3.336 kX.U. and a radius of curvature of 200 mm. The photometric measurements were performed in the same areas as for the determinations of calcium (Fig. 71 a—b). In equation (78) the correction factor in the numerator had a value of 0.95 and the denominator a value of 2330.

The results of the analyses are presented in Table XVII. The standard errors of the means of the densities were calculated, and the standard errors

of the amounts of calcium and phosphorus were obtained according to equation (78 a). The ratio between the amounts of calcium and phosphorus in the two Haversian systems was thus 1.7 and 1.9, respectively.

These analyses show that it is possible to determine the quantitative distributions of calcium and phosphorus in single Haversian systems. The experimental errors of the elementary analyses were about ± 3 per cent for ground slices with a thickness of about 10μ .

B. Determination of Calcium and Phosphorus in the Arterial Wall in Arteriosclerosis

The degenerative changes occurring in the arterial walls in arteriosclerosis consist of a fibrous thickening and deposition of hyaline and necrotic material, in which calcification takes place. The absorption of soft continuous roentgen radiation is rather high in such deposits, as is illustrated in the microradiogram in Fig. 72, registered at 1.5 kV across the roentgen tube. The difference between the absorption images of the arterial wall, registered on both sides of the K absorption edge of calcium (Fig. 73 a—b), and on both sides of the K absorption edge of phosphorus (Fig. 74 a—b), respectively, is conspicuous. The absorption images were registered at the same wavelengths as described in section A.

The amounts of calcium and phosphorus per unit area were determined in a homogeneous area within the calcification in the arterial wall. For calcium $(11.5 \pm 0.2) \mu\mu\text{g} \cdot \mu^{-2}$ was obtained, and the corresponding value for phosphorus was $(3.5 \pm 0.1) \mu\mu\text{g} \cdot \mu^{-2}$. Thus a ratio of 3.3 was obtained between the amounts of calcium and phosphorus in the calcified structure.

C. Determination of Calcium in a Renal Calculus

A microradiogram of a $\sim 10 \mu$ thick section of human kidney with a renal calculus is shown in Fig. 75. The absorption image was registered with continuous roentgen radiation generated at 1.5 kV. Microradiograms were also registered, utilizing monochromatic roentgen radiation on both sides of the K absorption edge of calcium (Fig. 76 a—b). For a homogeneous structure within the calculus the amount of calcium was determined, as described previously. A value of $(5.3 \pm 0.2) \mu\mu\text{g} \cdot \mu^{-2}$ was obtained.



Fig. 72. Microradiogram, $\times 265$, of a $\sim 10 \mu$ thick section of formalin fixed human art. femoral., registered at 1.5 kV (continuous radiation).

In the three different determinations of the amounts of calcium per unit area the absolute errors were of the same magnitude, i.e. about $0.2 \mu\text{g} \cdot \mu^{-2}$. From this value the smallest measurable amount of calcium per unit area can be calculated, provided the allowable relative experimental error is fixed.

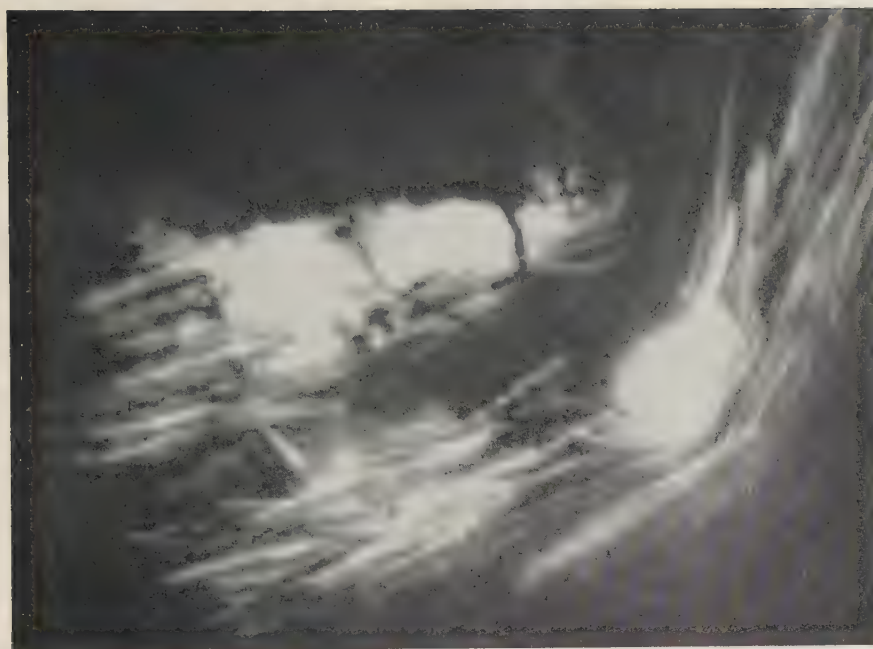


Fig. 73. Microradiogram, $\times 265$, of the same section as in Fig. 72.
a) Registered at ~ 3.03 kX.U. b) Registered at ~ 3.10 kX.U.



a



b

Fig. 74. Microradiogram, $\times 265$, of the same section as in Fig. 72.
a) Registered at 5.71 kX.U. b) Registered at 5.82 kX.U.

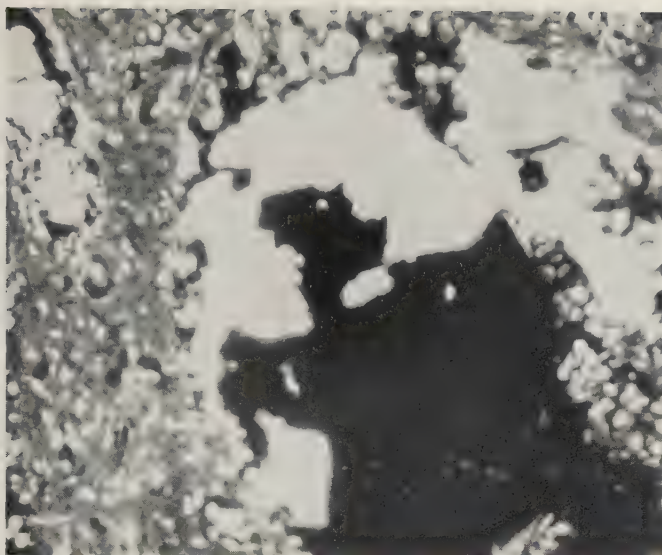


Fig. 75. Microradiogram, $\times 265$, of a $\sim 10 \mu$ thick section of formalin fixed human kidney with renal calculi, registered at 1.5 kV (continuous radiation).



a



b

Fig. 76. Microradiogram, $\times 265$, of the same section as in Fig. 75.
a) Registered at ~ 3.03 kX.U. b) Registered at ~ 3.10 kX.U.

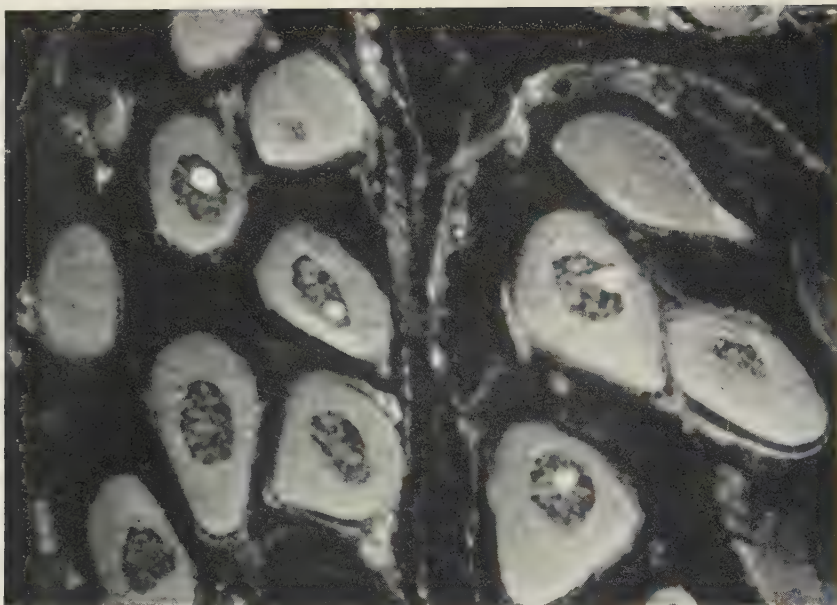


Fig. 77. Microradiogram, $\times 195$, of a $\sim 8 \mu$ thick section of sea urchin gonad fixed in Carnoy's solution, registered at 1.5 kV (continuous radiation).

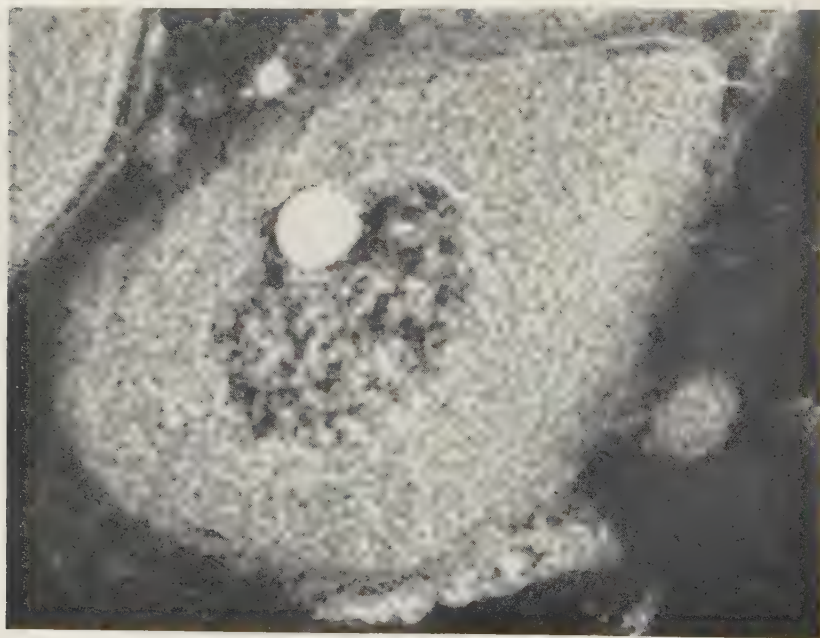


Fig. 78. Microradiogram, $\times 675$, of the same section as in Fig. 77, registered at 1.5 kV (continuous radiation).



Fig. 79. Microradiogram, $\times 675$, of the same section as in Fig. 77.
a) Registered at 5.71 kX.U. b) Registered at 5.82 kX.U.

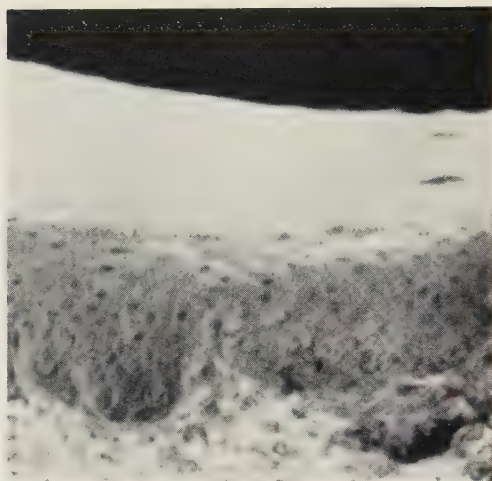
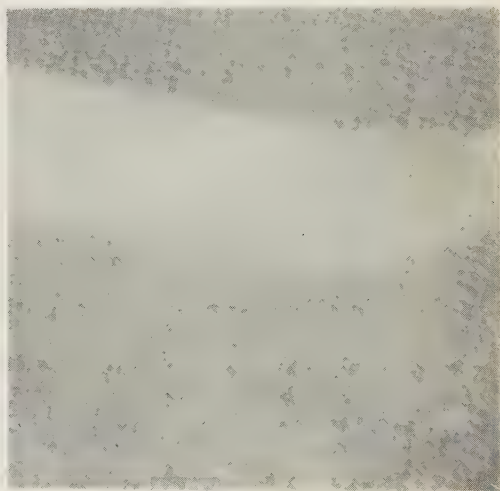


Fig. 80. Microradiogram, $\times 300$, of $\sim 10 \mu$ thick section of formalin fixed rat epidermis, registered at 1.5 kV (continuous radiation).



a



b

Fig. 81. Microradiogram, $\times 300$, of the same section as in Fig. 80.
a) Registered at 4.84 kX.U. b) Registered at 5.17 kX.U.

D. Determination of Phosphorus in Sea Urchin Eggs

A microradiogram of a $\sim 8 \mu$ thick section of a sea urchin gonad, fixed in Carnoy's solution is shown in Fig. 77, registered with continuous roentgen radiation, generated at 1.5 kV. As can be seen in Fig. 78, the total dry weight is homogeneously distributed in the cytoplasm of the egg, whereas the nuclear sap in this fixed material has a lower total dry weight, except for the nucleolus which has a high dry weight per unit area. Quantitative determinations revealed a total dry weight of $(3.5 \pm 0.1) \mu\text{ug} \cdot \mu^{-2}$ for the cytoplasm. The total dry weight of the nucleolus was about $8.3 \mu\text{ug} \cdot \mu^{-2}$. This high dry weight of the nucleolus indicates rather great variations of the thickness of the section and considerable shrinkage effects.

The absorption of monochromatic roentgen radiation at the wavelengths 5.712 kX.U. and 5.824 kX.U. was measured in the same cell by means of direct photometry of the enlarged images of the microradiograms (Fig. 79 a and b). The amount of phosphorus in the cytoplasm and in the nucleolus, calculated from the density values as described previously, was found to be 0.14 and $0.37 \mu\text{ug} \cdot \mu^{-2}$, respectively. The first value, however, is lower than the smallest amount that can be considered quantitatively measurable. Expressed in percentage of weight the amount of phosphorus in the nucleolus was found to be about 4.4 per cent.

This application illustrates the importance of correlating elementary analyses with total dry weight determinations in the same structure. In this case the amount of phosphorus in the nucleolus, expressed in percentage of weight, is independent of variations of the thickness of the section and of shrinkage effects.

E. Determination of the Amount of Sulphur in Rat Epidermis

In epidermis the corneal layer has a considerably higher dry weight than the spinous layer (Fig. 80). For the analyses of sulphur the characteristic roentgen emission lines $\text{Ru } L\alpha_1$ ($\lambda = 4.836 \text{ kX.U.}$) and $\text{Mo } L\beta_1$ ($\lambda = 5.166 \text{ kX.U.}$) were utilized. The K absorption edge of sulphur is situated at 5.008 kX.U. [13]. The same quartz crystal was utilized as for the determinations of phosphorus.

Fig. 81 a and b show the roentgen absorption images of rat epidermis, registered at the wavelengths given above. The amounts of sulphur were

calculated from the density values according to equation (78). The correction factor in the numerator had a value of 0.83 and the denominator a value of 1870.

In the *stratum corneum* the amount of sulphur was $(0.45 \pm 0.06) \mu\mu\text{g} \cdot \mu^{-2}$ and in the *stratum spinosum* $(0.29 \pm 0.05) \mu\mu\text{g} \cdot \mu^{-2}$. The ratio between the amounts of sulphur per unit area of the two layers was thus 1.6. This value is of about the same magnitude as the ratio between the dry weights of the two layers. Earlier histochemical analyses of sulphur [194] are therefore not correct.

General summary

PART I

1. The physical basis of quantitative roentgen absorption spectrophotometry is presented with special reference to the cytochemical applications.

The properties of the continuous roentgen spectrum are discussed. The different investigations of the distribution of the intensity in the continuous roentgen spectrum are surveyed with special reference to continuous roentgen spectra generated at low voltages.

The basic mechanism for the production of the characteristic roentgen emission spectra and the important properties of the roentgen emission lines are briefly discussed.

2. The general laws are given for the absorption of roentgen radiation, and the different coefficients utilized are defined. The negligible influence of the scattering process at wavelengths longer than 2.5 kX.U. is shown. The nature of the photoelectric absorption process is discussed with special reference to the absorption edges. The fine structure at the absorption edges and the influence of chemical combination upon the position of the absorption edges is surveyed. A review is given of different empirical absorption formulae and investigations of the magnitude of the absorption edges.

3. Different methods, utilized to obtain monochromatic roentgen radiation, are surveyed with special reference to methods applicable to soft roentgen rays. Bragg's law is given with correction for diffraction in higher orders. The advantage of bent crystals as compared with plane crystals is considered, and the method of bent crystal according to Johann is discussed.

4. Different methods to register the intensity of roentgen rays are discussed. The importance of photographic procedures at the cytochemical applications of roentgen absorption spectrophotometry is stressed.

The photographic action of roentgen rays is discussed with special reference to the density as a function of the exposure. The ionization chamber, the proportional counter, the Geiger counter, and the scintillation counter are briefly described.

PART II

5. The biologic applications of microradiography is reviewed with special regard to the quantitative interpretation of roentgen absorption images of biologic objects.

6. The extended theoretical basis of total dry weight determinations is presented. The systematic errors of total dry weight determinations, utilizing monochromatic roentgen radiation, are first calculated for different elements and common organic compounds in biologic objects. The calculations are then extended to the case when a continuous, filtered roentgen spectrum, generated at 1.5 kV, is utilized. Different distributions of the intensity in the continuous spectrum are considered. Formulae for the calculation of weight per unit area are given, when a reference system is utilized.

7. An equipment for total dry weight determinations, utilizing extremely soft roentgen radiation, has been constructed. The roentgen tube is described in detail, and constructional drawings are presented.

8. The experimental procedure for total dry weight determinations is described. The procedure of making the reference system and its accuracy are discussed. The different factors contributing to the error of the photometric determinations are considered.

The spatial resolving power of the microradiographic technique is discussed. Suitable developing conditions are determined from relative, objective measurements of the granularity of the fine-grained photographic emulsions, utilizing the standard deviations of the photo-currents.

Series of microradiograms are presented, illustrating the greater quantitative resolving power, when the utilized continuous roentgen spectrum is generated at low voltages.

The total resolving power and the combined experimental error are discussed. Total dry weight determinations of squamous epithelium could be performed with an experimental error of about ± 5 per cent.

9. Some applications of total dry weight determinations of cytologic structures are presented. The smallest measured volumes of organic material are about $5 \mu^3$. The dry weight of individual cells has been determined, and the values agree with the results from microchemical analyses.

PART III

10. Quantitative elementary analyses by roentgen ray methods are surveyed with special reference to biologic applications, utilizing the characteristic discontinuities of the values of the mass photoelectric absorption coefficients for different elements.

11. The theoretical basis for elementary analyses by roentgen absorption spectrophotometry is described, and the accuracy and the sensitivity of the method are discussed.

12. A high vacuum roentgen ray spectrophotometer with bent crystal has been constructed in order to obtain high intensities of monochromatic roentgen radiation. Constructional drawings and a description of the spectrophotometer are presented. The radius of curvature of the crystal is 200 or 385 mm, and the opening 20 mm. A wiring diagram of the high voltage supply is given.

13. The adjustment of the spectrophotometer and errors of the bent crystal technique are discussed.

Registering with a Geiger counter and a photographic-photometric procedure are presented. A homogeneous distribution of the intensity of monochromatic roentgen radiation is obtained, when the microradiograms are registered at a distance behind the slit, placed on the Rowland circle.

The density as a function of the exposure is determined for Kodak High Resolution plates at 2.74 and 5.71 kX.U. The errors of the photometric technique are discussed.

14. Quantitative determinations on a cellular level of calcium, phosphorus, and sulphur are presented, utilizing the photographic-photometric technique. The absolute errors are between ± 0.15 and $\pm 0.2 \mu\mu\text{g} \cdot \mu^{-2}$ at determinations of calcium and between ± 0.06 and $\pm 0.1 \mu\mu\text{g} \cdot \mu^{-2}$ at determinations of phosphorus and sulphur in sections of biologic objects with a thickness of about 10μ .

References

INTRODUCTION and PART I

- [1] ANDREWS, C. L.: The absorption of X-Rays of Wave-Length $1.5 \leq \lambda \leq 8.3 \text{ \AA}$. *Phys. Rev.*, *54* (1938), 994—999.
- [2] ARENDS, E.: Der Ausbeutekoeffizient u der charakteristischen K-Strahlung und das Verhältnis von Luftionisation zu Energie der Röntgenstrahlen. *Ann. Physik*, *22* (1935), 281—310.
- [3] BANDOPADHYAYA, G. B., & MAITRA, A. T.: Absorption of Soft X-Rays in Aluminium. *Phil. Mag.*, *21* (1936), 869—880.
- [4] BELL, G. E.: The Photographic Action of X-Rays. *Brit. J. Radiol.*, *9* (1936), 578—605.
- [5] BIERMANN, H.-H.: Die Massenschwächungskoeffizienten monochromatischer Röntgenstrahlen für Cellophan, Al, Se, Ag, Cd, Sn, Sb und Te bis 10 ÅE. *Ann. Physik*, *26* (1936), 740—760.
- [6] BOUWERS, A.: Über die Schwärzung der photographischen Platte durch Röntgenstrahlen und ihre Anwendung zur Intensitätsmessung. *Z. Physik*, *14* (1923), 374—382.
- [7] BRINDLEY, G. W., & SPIERS, F. W.: Experiments on the Photographic Effect of X-Rays. *Phil. Mag.*, *16* (1933), 686—691.
- [8] BROILI, H., & KIESSIG, H.: Das photographische Schwärzungsgesetz bei ultra-weicher Röntgenstrahlung. *Z. Physik*, *87* (1933—34), 425—431.
- [9] BROMLEY, D., & HERZ, R. H.: Quantum Efficiency in Photographic X-Ray Exposures. *Proc. Phys. Soc. (London)*, *B*, *63* (1950), 90—106.
- [10] BUSSE, W.: Das photographische Schwärzungsgesetz für homogene Röntgenstrahlen. *Z. Physik*, *34* (1925), 11—14.
- [11] CASPERSSON, T.: Über den chemischen Aufbau der Strukturen des Zellkernes. *Skand. Arch. Physiol., Suppl.* *8* (1936).
- [12] — *Cell Growth and Cell Function*. W. W. Norton and Company, Inc., New York (1950).
- [13] CAUCHOIS, Y., & HULUBEI, H.: *Constantes sélectionnées longueurs d'onde des émissions X et des discontinuités d'absorption X*. Tables de constantes et données numériques, 1. Hermann & C^{ie}, Éditeurs, Paris (1947).
- [14] CHARLESBY, A.: The Action of Electrons and X-Rays on Photographic Emulsions. *Proc. Phys. Soc. (London)*, *52* (1940), 657—700.
- [15] CLARK, G. L.: *Applied X-Rays*. McGraw-Hill Book Company, Inc., New York, 4th Ed. (1955).
- [16] COMPTON, A. H., & ALLISON, S. K.: *X-Rays in Theory and Experiment*. D. van Nostrand Company, Inc., New York, 2nd Ed. (1935).
- [17] COSTER, D., & KIESTRA, S.: On the Empty Electron Bands of Lowest Energy of the Transition Metals Mn and Fe and Their Oxides. *Physica*, *14* (1948), 175—188.

- [18] —, — On the Fine Structure Near the FeK-Absorption Edges in the Various Oxides of Fe. *Phil. Mag.*, 41 (1950), 144—151.
- [19] CRONE, W.: Der Nutzeffekt der Röntgen-K-Fluoreszenzstrahlung bei den Elementen C, N, O, Ne. *Ann. Physik*, 27 (1936), 405—420.
- [20] CURRAN, S. C.: The New Counters. *Science Progr.*, 42 (1954), 32—45.
- [21] —, ANGUS, J., & COCKROFT, A. L.: Investigation of Soft Radiations by Proportional Counters. I. *Phil. Mag.*, 40 (1949), 36—52.
- [22] DERSHEM, E., & SCHEIN, M.: The Absorption of the K α Line of Carbon in Various Gases and Its Dependence upon Atomic Number. *Phys. Rev.*, 37 (1931), 1238—1245.
- [23] —, — Über die Reflexion und Absorption langwelliger Röntgenstrahlen. *Z. Physik*, 75 (1932), 395—414.
- [24] DuMOND, J. W. M.: A Solid-State Interpretation of the Structure Near the Limit of the Continuous X-Ray Spectrum. *Phys. Rev.*, 72 (1947), 276—283.
- [25] — & COHEN, E. R.: Least-Squares Adjustment of the Atomic Constants, 1952. *Revs Mod. Phys.*, 25 (1953), 691—708.
- [26] — & KIRKPATRICK, H. A.: The Multiple Crystal X-Ray Spectrograph. *Rev. Sci. Instr.*, 1 (1930), 88—105.
- [27] EGGERT, J., & NODDACK, W.: Über die Quantenausbeute bei der Wirkung von Röntgenstrahlen auf Silberbromid. *Z. Physik*, 43 (1927), 222—229.
- [28] —, — Über die Quantenausbeute bei der Wirkung von Röntgenstrahlen auf Silberbromid. II. *Z. Physik*, 51 (1928), 796—804.
- [29] ENGSTRÖM, A.: Quantitative Micro- and Histochemical Elementary Analysis by Roentgen Absorption Spectrography. *Acta Radiol., Suppl.* 63 (1946).
- [30] — & LINDSTRÖM, B.: A Method for the Determination of the Mass of Extremely Small Biological Objects. *Biochim. et Biophys. Acta*, 4 (1950), 351—373.
- [31] FAESSLER, A., & GOEHRING, M.: Röntgenspektrum und Bindungszustand. Die K α -Fluoreszenzstrahlung des Schwefels. *Naturwissenschaften*, 39 (1952), 169—177.
- [32] FRIEDMAN, H.: Applications of Electronic Methods to the Measurement of X-Ray and Gamma Ray Intensities. *Ind. Radiography Non-Destructive Testing*, 6, No. 1 (1947), 9—20.
- [33] FRIEDRICH, W., & KOCH, P. P.: Über Methoden zur photographischen Spektralphotometrie der Röntgenstrahlen. *Ann. Physik*, 45 (1914), 399—418.
- [34] GEIGER, H.: *Röntgenstrahlung ausschliesslich Röntgenoptik. Handbuch der Physik* (GEIGER & SCHEEL), Vol. XXIII:2. Verlag von Julius Springer, Berlin, 2nd Ed. (1933).
- [35] GLOCKER, R.: Über das Grundgesetz der physikalischen Wirkungen von Röntgenstrahlen verschiedener Wellenlänge. *Z. Physik*, 43 (1927), 827—838; 46 (1928), 764.
- [36] — Über die Gesetzmässigkeiten der physikalischen und chemischen Wirkung der Röntgenstrahlen. *Z. tech. Phys.*, 9 (1928), 201—207.
- [37] — *Materialprüfung mit Röntgenstrahlen*. Springer-Verlag, Berlin, 3rd Ed. (1949).
- [38] — & TRAUB, W.: Das photographische Schwärzungsgesetz der Röntgenstrahlen. *Physik. Z.*, 22 (1921), 345—352.

- [39] GROSSKURTH, K.: Neubestimmung der Massenschwächungskoeffizienten monochromatischer Röntgenstrahlen für 16 Elemente und Paraffin zwischen 0.128 und 2.5 ÅE. *Ann. Physik*, 20 (1934), 197—232.
- [40] GÜNTHER, P., & TITTEL, H.: Die Bildung von Silber in der photographischen Schicht unter dem Einfluss von Röntgenstrahlen. *Z. Elektrochem.*, 39 (1933), 646—655.
- [41] HAAS, M.: Der Nutzeffekt der Röntgen-K-Fluoreszenzstrahlung bei leichten Elementen. *Ann. Physik*, 16 (1933), 473—488.
- [42] HANSEN, H.: Die Schwächung monochromatischer Röntgenstrahlen in flüssigem und gasförmigem CS₂, CH₂Cl₂ und C₂H₅Br sowie in gasförmigem CH₃J zwischen 0.1623 und 1.933 Å. *Ann. Physik*, 35 (1939), 524—546.
- [43] HILL, R. D., CHURCH, E. L., & MIHELICH, J. W.: The Determination of Gamma-Ray Energies from Beta-Ray Spectroscopy and a Table of Critical X-Ray Absorption Energies. *Rev. Sci. Instr.*, 23 (1952), 523—528.
- [44] HIRSH, F. R., JR.: The Blackening of Photographic Plates by Long Wavelength X-Rays. *J. Opt. Soc. Amer.*, 25 (1935), 229—230.
- [45] — The Blackening of Photographic Plates by Long Wave-Length X-Rays, II. *J. Opt. Soc. Amer.*, 28 (1938), 463—465.
- [46] HODGMAN, C. D.: *Handbook of Chemistry and Physics*. Chemical Rubber Publishing Co., Cleveland, 33rd Ed. (1951—52).
- [47] HOERLIN, H.: The Photographic Action of X-Rays in the 1.3 to 0.01 Å Range. *J. Opt. Soc. Amer.*, 39 (1949), 891—897.
- [48] — Die Quantenausbeute der photographischen Wirkung der Röntgenstrahlen als Funktion der Wellenlänge und der Sensibilisierung. *Z. Naturforsch.*, 6 a (1951), 344—349.
- [49] HOLWECK, F.: *De la lumière aux rayons X*. Recueil des conférences-rapports de documentation sur la physique, Vol. 13. Les presses universitaires de France, Paris (1927).
- [50] JOHANN, H. H.: Die Erzeugung lichtstarker Röntgenspektren mit Hilfe von Konkavkristallen. *Z. Physik*, 69 (1931), 185—206.
- [51] JOHANSSON, T.: Über ein neuartiges, genau fokussierendes Röntgenspektrometer. *Z. Physik*, 82 (1933), 507—528.
- [52] JÖNSSON, E.: *Absorptionsmessungen im langwelligen Röntgengebiet und Gesetze der Absorption*. Dissertation, Uppsala Univ. Årsskr. (1928).
- [53] KIRCHNER, F.: *Allgemeine Physik der Röntgenstrahlen. Handbuch der Experimentalphysik* (WIEN & HARMS). Vol. XXIV:1. Akademische Verlagsgesellschaft m.b.H., Leipzig (1930).
- [54] KIRKPATRICK, P.: On the Theory and Use of Ross Filters. *Rev. Sci. Instr.*, 10 (1939), 186—191.
- [55] — Theory and Use of Ross Filters. II. *Rev. Sci. Instr.*, 15 (1944), 223—229.
- [56] KLEIN, O., & NISHINA, Y.: Über die Streuung von Strahlung durch freie Elektronen nach der neuen relativistischen Quantendynamik von Dirac. *Z. Physik*, 52 (1929), 853—868.
- [57] KORFF, S. A., & PRESENT, R. D.: On the Role of Polyatomic Gases in Fast Counters. *Phys. Rev.*, 65 (1944), 274—282.
- [58] KORNFELD, G.: The Latent Image Produced by X-Rays. *The Theory of the Photographic Process* (MEES), Pp. 327—344. The MacMillan Company, New York, 2nd Ed. (1954).

- [59] KRAMERS, H. A.: On the Theory of X-Ray Absorption and of the Continuous X-Ray Spectrum. *Phil. Mag.*, 46 (1923), 836—871.
- [60] DE L. KRONIG, R.: Zur Theorie der Feinstruktur in den Röntgenabsorptionsspektren. *Z. Physik*, 70 (1931), 317—323.
- [61] KULENKAMPFF, H.: Über das kontinuierliche Röntgenspektrum. *Ann. Physik*, 69 (1922), 548—596.
- [62] — & SCHMIDT, L.: Die Energieverteilung im Spektrum der Röntgen-Bremsstrahlung. *Ann. Physik*, 43 (1943), 494—512.
- [63] KURTZ, H.: Die Absorption der Kohlenstoff-K-Strahlung in C, N und O. *Ann. Physik*, 85 (1928), 529—551.
- [64] KÜSTNER, H.: Die Erzeugung intensiver monochromatischer Röntgenstrahlen mit Hilfe technischer Röhren ohne Spektralapparat. *Z. Physik*, 70 (1931), 324—347.
- [65] — Einige Anwendungen des Filterdifferenzverfahrens zur Erzeugung monochromatischer Röntgenstrahlen. *Z. Physik*, 70 (1931), 468—491.
- [66] — Eine Verbesserung des Filterdifferenzverfahrens zur Erzielung intensiver monochromatischer Röntgenstrahlen. *Z. Physik*, 77 (1932), 52—59.
- [67] — Der Absorptionssprung an den L-Bandkanten der Schwerelemente. *Physik, Z.*, 33 (1932), 46—49.
- [68] — & ARENDS, E.: Ausbeutekoeffizienten, Intensitätsverhältnisse und Absorptionswahrscheinlichkeiten in der L-Serie der Schwerelemente. *Ann. Physik*, 22 (1935), 443—472.
- [69] — & GROSSKURTH, K.: Die Schwächung von Röntgenstrahlen beim Durchgang durch Materie. *Strahlentherapie*, 52 (1935), 115—136.
- [70] LAUBERT, S.: Die Massenschwächungs-, Photoabsorptions- und Streukoeffizienten monochromatischer Röntgenstrahlen von Ni(28), Cu(29), Ag(47), Cd(48), Sn(50), Ta(73), W(74), Ir(77), Pt(78), Au(79) und Pb(82). *Ann. Physik*, 40 (1941), 553—578.
- [71] LAY, H.: Die Fluoreszenzausbeute des L-Gebiets. *Z. Physik*, 91 (1934), 533—550.
- [72] LIEBSON, S. H., & FRIEDMAN, H.: Self-Quenching Halogen-Filled Counters. *Rev. Sci. Instr.*, 19 (1948), 303—306.
- [73] LINDH, A. E.: *Röntgenspektroskopie. Handbuch der Experimentalphysik* (WIEN & HARMS), Vol. XXIV: 2. Akademische Verlagsgesellschaft m.b.H., Leipzig (1930).
- [74] MAGNUSSON, T.: Investigations into Absorption Spectra in the Extremely Soft X-Ray Region. *Nova Acta Regiae Soc. Sci. Upsalensis, Ser. 4, Vol. 11, No. 3* (1938).
- [75] MARSHALL, F.-H., COLTMAN, J. W., & BENNETT, A. I.: The Photo-Multiplier Radiation Detector. *Rev. Sci. Instr.*, 19 (1948), 744—770.
- [76] —, & HUNTER, L. P.: The Photomultiplier X-Ray Detector. *Rev. Sci. Instr.*, 18 (1947), 504—513.
- [77] MARTIN, L. H., & LANG, K. C.: X-Ray Absorption Coefficients in the Range 0.3 to 2.0 Å. *Proc. Roy. Soc. (London), A*, 137 (1932), 199—216.
- [78] MÜLBACH, E.: Untersuchungen über die Gültigkeit des Bunsen-Roscoeschen Gesetzes für die photographische Wirkung und den Verlauf der Schwärzungskurven langwelliger Röntgenstrahlen. *Z. wiss. Phot.*, 36 (1937), 269—282.

- [79] MÜLLER, I.: Über den Massenschwächungskoeffizienten des Wassers und wässriger Lösungen von Verbindungen der Elemente Se, Br, Cd, J, Ba, Ce und Tl. *Ann. Physik*, 32 (1938), 625—639.
- [80] NEFF, H.: Die Energieverteilung im kontinuierlichen Röntgenspektrum zwischen 1 und 2 kV. *Z. Physik*, 131 (1951), 1—9.
- [81] NICHOLAS, W. W.: Continuous Spectrum X-Rays from Thin Targets. *Bur. Standards J. Research*, 2 (1929), 837—870.
- [82] ORTNER, G.: Röntgenspektroskopie mit gebogenen Kristallen. *Österr. Chem.-Ztg*, 44 (1941), 110—114.
- [83] PELC, S. R.: The Photographic Action of X-Rays. *Proc. Phys. Soc. (London)*, 57 (1945), 523—534.
- [84] RINDFLEISCH, H.: Über den K-Sprung von $Al_{(13)}$ bis $W_{(74)}$. *Ann. Physik*, 28 (1937), 409—437.
- [85] SANNER, V. H.: *Die K-Emissions- und Absorptionsspektren der Elemente Kalium bis Kupfer und einiger ihrer Oxyde*. Dissertation, Uppsala, (1941).
- [86] SCHULZ, K.: Die Massenschwächungskoeffizienten monochromatischer Röntgenstrahlen für Se, Ag, Cd, Te, Au, Pb und Bi bis etwa 2 ÅE. *Ann. Physik*, 27 (1936), 1—14.
- [87] SEEMANN, H. E.: Spectral Sensitivity of Two Commercial X-Ray Films between 0.2 and 2.5 Angstroms. *Rev. Sci. Instr.*, 21 (1950), 314—322.
- [88] SIEGBAHN, M.: *Spektroskopie der Röntgenstrahlen*. Verlag von Julius Springer, Berlin, 2nd Ed. (1931).
- [89] SILBERSTEIN, L., & TRIVELLI, A. P. H.: The Quantum Theory of X-Ray Exposures on Photographic Emulsions. *Phil. Mag.*, 9 (1930), 787—800.
- [90] SKINNER, H. W. B.: The Soft X-Ray Spectroscopy of the Solid State. *Repts Progr. in Phys.*, 5 (1938), 257—283.
- [91] SPROULL, W. T.: *X-Rays in Practice*. McGraw-Hill Book Company, Inc., New York, 1st Ed. (1946).
- [92] STEPHENSON, S. T., & MASON, F. D.: Continuous X-Ray Spectrum from 8 Å to 14 Å. *Phys. Rev.*, 75 (1949), 1711—1716.
- [93] TELLEZ-PLASENCIA, H.: Théorie générale des méthodes de mesure des rayons X. *J. phys. radium*, 9 (1948), 230—235.
- [94] — Formules d'interpolation pour déterminer les valeurs numériques de quelques constantes atomiques. *J. phys. radium*, 10 (1949), 14—19.
- [95] TYRÉN, F.: Precision Measurements of Soft X-Rays with Concave Grating. *Nova Acta Regiae Soc. Sci. Upsalensis, Ser. 4, Vol. 12, No. 1* (1940).
- [96] VICTOREEN, J. A.: Probable X-Ray Mass Absorption Coefficients for Wave-Lengths Shorter Than the K Critical Absorption Wave-Length. *J. Appl. Phys.*, 14 (1943), 95—102.
- [97] — Atomic Absorption Coefficient for X-Rays. *Phys. Rev.*, 72 (1947), 869.
- [98] — The Absorption of Incident Quanta by Atoms as Defined by the Mass Photoelectric Absorption Coefficient and the Mass Scattering Coefficient. *J. Appl. Phys.*, 19 (1948), 855—860.
- [99] — The Calculation of X-Ray Mass Absorption Coefficients. *J. Appl. Phys.*, 20 (1949), 1141—1147.
- [100] — Roentgen Rays: Measurement of Quality. *Medical Physics* (GLASSER), Vol. II, Pp. 887—894. The Year Book Publishers, Inc., Chicago (1950).
- [101] DE WAARD, R. H.: The Distribution of Energy in Continuous X-Ray Spectra

- Corresponding to Different Forms of High Tension, and the Influence of Filtering. I. *Koninkl. Ned. Akad. Wetenschap. Proc.*, 49 (1946), 944—954.
- [102] — The Distribution of Energy in Continuous X-Ray Spectra Corresponding to Different Forms of High Tension, and the Influence of Filtering. II. *Koninkl. Ned. Akad. Wetenschap. Proc.*, 49 (1946), 1011—1015.
- [103] — A System of Formulae and Curves Bearing on the Distribution of Energy in the Continuous Roentgen Spectrum. *Acta Radiol.*, 28 (1947), 37—48.
- [104] WALTER, B.: Über die besten Formeln zur Berechnung der Absorption der Röntgenstrahlen in einem beliebigen Stoff. *Fortschr. Gebiete Röntgenstrahlen*, 35 (1927), 929—947, 1308—1310.
- [105] WARMOLTZ, N.: Geiger-Müller Counters. *Philips Tech. Rev.*, 13 (1951—52), 282—292.
- [106] WOERNLE, B.: Die Absorption langwelliger Röntgenstrahlen von 2—10 ÅE in leichten Elementen. *Ann. Physik*, 5 (1930), 475—506.
- [107] WOLF, M.: Die Absorptionskoeffizienten für Röntgenstrahlen in der Umgebung der L-Kanten bei den Elementen Au, Pt und Ag. *Ann. Physik*, 16 (1933), 973—984.

PART II

- [108] ABDERHALDEN, E.: *Biochemisches Handlexikon*. Verlag von Julius Springer, Berlin, Band 4 (1911), Band 14 (1933).
- [109] AMPRINO, R., & ENGSTRÖM, A.: Studies on X-Ray Absorption and Diffraction of Bone Tissue. *Acta Anat.*, 15 (1952), 1—22.
- [110] BARCLAY, A. E.: Micro-Arteriography. *Brit. J. Radiol.*, 20 (1947), 394—404.
- [111] — Demonstration of Iron in Cells. A Preliminary Note. *Brit. J. Radiol.*, 22 (1949), 268—269.
- [112] — *Micro-Arteriography*. Blackwell Scientific Publication, Oxford (1951).
- [113] BELLMAN, S.: Microangiography. *Acta Radiol., Suppl.* 102 (1953).
- [114] — & ENGSTRÖM, A.: Microangiography. *Acta Radiol.*, 38 (1952), 98—110.
- [115] BOHATYRTSCHUK, F.: Über Ergebnisse der Mikroröntgenographie. *Acta Radiol.*, 25 (1944), 351—365.
- [116] BOURGHARDT, S., BRATTGÅRD, S.-O., HYDÉN, H., JIEWERTZ, B., & LARSSON, S.: A Microphotometer for Microradiography. *J. Sci. Instr.*, 30 (1953), 464—466.
- [117] BRATTGÅRD, S.-O.: The Importance of Adequate Stimulation for the Chemical Composition of Retinal Ganglion Cells during Early Post-Natal Development. *Acta Radiol., Suppl.* 96 (1952).
- [118] — & HALLÉN, O.: X-Ray Microradiography as a Quantitative Method. *Biochim. et Biophys. Acta*, 9 (1952), 488—495.
- [119] —, — & HYDÉN, H.: A Critical Evaluation of X-Ray Microradiography. *Biochim. et Biophys. Acta*, 10 (1953), 486—487.
- [120] —, —, — The Elimination of Errors in Roentgen Microradiography. *Acta Radiol.*, 39 (1953), 494—498.
- [121] — & HYDÉN, H.: Mass, Lipids, Pentose Nucleoproteins and Proteins Determined in Nerve Cells by X-Ray Microradiography. *Acta Radiol., Suppl.* 94 (1952).
- [122] —, — The Composition of the Nerve Cell Studied with New Methods. *Intern. Rev. Cytol.*, 3 (1954), 455—476.

- [123] CASTEL, P., LAMARQUE, P., & TURCHINI, J.: Historiographie et localisation histologique de substances médicamenteuses ou toxiques à poids atomique élevé. *Compt. rend. soc. biol.*, 123 (1936), 1051—1052.
- [124] CLARK, G. L.: Medical, Biological and Industrial Applications of Monochromatic Radiography and Microradiography. *Radiology*, 49 (1947), 483—495.
- [125] CLEMMONS, J. J., & APRISON, M. H.: An Improved Historiographic Apparatus. *Rev. Sci. Instr.*, 24 (1953), 444—454.
- [126] —, — & ANGEVINE, D. M.: An Improved Historiographic Apparatus and Its Application. *Federation Proc.*, 10 (1951), 352.
- [127] —, LALICH, J. J., & ANGEVINE, D. M.: The Technic of Quantitative Historiography. *Lab. Investigation*, 3 (1954), 19—32.
- [128] — & WEBSTER, T. C.: An Accurate Reference System for Historiography. *Biochim. et Biophys. Acta*, 11 (1953), 464—470.
- [129] COMBÉE, B., & ENGSTRÖM, A.: A New Device for Microradiography and a Simplified Technique for the Determination of the Mass of Cytological Structures. *Biochim. et Biophys. Acta*, 14 (1954), 432—434.
- [130] COSSLETT, V. E., & NIXON, W. C.: X-Ray Shadow Microscope. *Nature*, 168 (1951), 24—25.
- [131] —, — X-Ray Shadow Microscopy. *Nature*, 170 (1952), 436—438.
- [132] —, — An Experimental X-Ray Shadow Microscope. *Proc. Roy. Soc. (London)*, B, 140 (1952—53), 422—431.
- [133] —, — The X-Ray Shadow Microscope. *J. Appl. Phys.*, 24 (1953), 616—623.
- [134] — & PEARSON, H. E.: An Improved X-Ray Shadow Projection Microscope. *J. Sci. Instr.*, 31 (1954), 255—257.
- [135] DAUVILLIER, A.: Réalisation de la microradiographie intégrale. *Compt. rend.*, 190 (1930), 1287—1289.
- [136] DJURLE, E., & HALLÉN, O.: A Double-Slit Interferometer for the Determination of the Thickness of the Reference System Used in X-Ray Microradiography. *Exptl Cell Research*, 5 (1953), 301—310.
- [137] EHRENBERG, W., & SPEAR, W. E.: X-Ray Micro-Radiography of Biological Specimens. *Nature*, 168 (1951), 513—514.
- [138] ENGFELDT, B.: Biophysical Studies on Bone Tissue VI. Biophysical Studies on Bone Tissue from Osteogenic Sarcoma. *Cancer*, 7 (1954), 815—825.
- [139] ENGSTRÖM, A.: Microradiography. *Acta Radiol.*, 31 (1949), 503—521.
- [140] — Use of Soft X-Rays in the Assay of Biological Material. *Progr. Biophys. and Biophys. Chem.*, 1 (1950), 164—196.
- [141] — & GLICK, D.: The Mass of Gastric Mucosa Cells Measured by X-Ray Absorption. *Science*, 111 (1950), 379—380.
- [142] — & LINDSTRÖM, B.: A New Method for Determining the Weight of Cellular Structures. *Nature*, 163 (1949), 563—564.
- [143] —, — The Properties of Fine-Grained Photographic Emulsions Used for Microradiography. *Acta Radiol.*, 35 (1951), 33—44.
- [144] — & LÜTHY, H.: The Distribution of Mass and Lipids in the Single Nerve Fiber. *Exptl Cell Research*, 1 (1950), 81—91.
- [145] — & RUCH, F.: Distribution of Mass in Salivary Gland Chromosomes. *Proc. Natl Acad. Sci. U. S.*, 37 (1951), 459—461.

- [146] — & WEGSTEDT, L.: Equipment for Microradiography with Soft Roentgen Rays. *Acta Radiol.*, 35 (1951), 345—355.
- [147] FITZGERALD, P. J., & ENGSTRÖM, A.: The Use of Ultraviolet-Microscopy, Roentgen-Ray-Absorption, and Radioautographic Techniques in the Study of Neoplastic Disease. *Cancer*, 5 (1952), 643—677.
- [148] FLASCHENTRÄGER, B., & LEHNARTZ, E.: *Physiologische Chemie*. Band I. *Die Stoffe*. Springer-Verlag, Berlin (1951).
- [149] GOBY, P.: Une application nouvelle des rayons X: la microradiographie. *Compt. rend.*, 156 (1913), 686—688.
- [150] — Micro-Radiography. *Arch. Roentg. Ray, London*, 18 (1913), 247—250.
- [151] HALLÉN, O.: A Simplified Method for the Preparation of the Reference System Used in X-Ray Microradiography. *Exptl Cell Research*, 4 (1953), 494—495.
- [152] — & INGELSTAM, E.: Determination by Interferometric Methods of the Thickness of the Reference System Used in Microradiography. *Exptl Cell Research*, 3 (1952), 248.
- [153] HEDBERG, E.: The Chemical Composition of the Human Ovarian Oocyte. *Acta Endocrinol., Suppl.* 15 (1953).
- [154] HIGGINS, G. C.: The Structure of the Developed Image. *The Theory of the Photographic Process* (MEES), Pp. 970—1046. The MacMillan Company, New York, 2nd Ed. (1954).
- [155] HILDITCH, T. P.: *The Chemical Constitution of Natural Fats*. Chapman and Hall Ltd., London, 2nd Ed. (1947).
- [156] KIRKPATRICK, P.: An Approach to X-Ray Microscopy. *Nature*, 166 (1950), 251—253.
- [157] — & BAEZ, A. V.: Formation of Optical Images by X-Rays. *J. Opt. Soc. Amer.*, 38 (1948), 766—774.
- [158] KOHLRAUSCH, F.: *Praktische Physik*. Band 1. B. G. Teubner Verlagsgesellschaft, Stuttgart, 20th Ed. (1955).
- [159] LAMARQUE, P.: Historadiography; New Application of X-Rays. *Radiology*, 27 (1936), 563—568.
- [160] — Historadiography. *Brit. J. Radiol.*, 11 (1938), 425—435.
- [161] — & TURCHINI, J.: Premiers résultats généraux obtenus par la technique de l'historadiographie. Degré de perméabilité des constituants tissulaires aux rayons X mous de $\lambda = 2.5 \text{ \AA}$. *Compt. rend. soc. biol.*, 122 (1936), 294—295.
- [162] LANGE, P. W., & ENGSTRÖM, A.: Determination of Thickness of Microscopic Objects. *Lab. Investigation*, 3 (1954), 116—131.
- [163] LINDSTRÖM, B.: The Accuracy of Cytologic Mass Determination by Roentgen Absorption. *Acta Radiol.*, 38 (1952), 355—360.
- [164] — The Reference System and Its Influence on the Accuracy of Cytological Mass Determination by X-Rays. *Biochim. et Biophys. Acta*, 10 (1953), 186—187.
- [165] — Absorption Spectrophotometry with Extremely Soft X-Rays for the Quantitative Determination of the Dry Weight of Cytological Structures. *Exptl Cell Research*, 6 (1954), 537—539.
- [166] — & MOBERGER, G.: Studies on the Quantitative Distribution of the Dry Weight in Squamous Epithelium during Carcinogenesis. *Exptl Cell Research*, 6 (1954), 540—542.

- [167] —, — The Distribution of the Dry Weight in Squamous Epithelium during Carcinogenesis Studied by Means of Quantitative Roentgen Absorption Spectrophotometry. *Exptl Cell Research*, in press.
- [168] MITCHELL, G. A. G.: Microradiographic Demonstration of Tissues Treated by Metallic Impregnation. *Nature*, 165 (1950), 429—430.
- [169] — Microradiography in Biology. *Brit. J. Radiol.*, 24 (1951), 110—117.
- [170] MOBERGER, G.: Malignant Transformation of Squamous Epithelium. *Acta Radiol., Suppl.* 112 (1954).
- [171] — & ENGSTRÖM, A.: Historadiographic Studies on Normal, Hyperplastic and Cancerous Epidermis. *J. Invest. Dermatol.*, 22 (1954), 477—491.
- [172] —, LINDSTRÖM, B., & ANDERSSON, L.: Freeze-Drying with a Modified Glick-Malmström Apparatus. *Exptl Cell Research*, 6 (1954), 228—237.
- [173] NURNBERGER, J., ENGSTRÖM, A., & LINDSTRÖM, B.: A Study of the Ventral Horn Cells of the Adult Cat by Two Independent Cytochemical Micro-absorption Techniques. *J. Cellular Comp. Physiol.*, 39 (1952), 215—254.
- [174] PATTEE, H. H., JR.: The Compound X-Ray Microscope. *Phys. Rev.*, 85 (1952), 764.
- [175] — The Scanning X-Ray Microscope. *J. Opt. Soc. Amer.*, 43 (1953), 61—62.
- [176] PRINCE, E.: The Resolving Power of an X-Ray Microscope. *J. Appl. Phys.*, 21 (1950), 698.
- [177] SIEVERT, R. M.: Two Methods of Roentgen Micro-Photography. *Acta Radiol.*, 17 (1936), 299—309.
- [178] SISSONS, H. A.: Microradiography of Bone. *Brit. J. Radiol.*, 23 (1950), 2—7.
- [179] STEIN, W. H., & MILLER, E. G., JR.: The Composition of Elastin. *J. Biol. Chem.*, 125 (1938), 599—614.
- [180] TRUETA, J., BARCLAY, A. E., FRANKLIN, K. J., DANIEL, P. M., & PRICHARD, M. M. L.: *Studies of the Renal Circulation*. Blackwell Scientific Publication, Oxford (1947).
- [181] TURCHINI, J.: L'historadiographie. — Applications. *Bull. histol. appl. physiol. et pathol. et tech. microscop.*, 14 (1937), 17—28.
- [182] WILSDORF, H.: Röntgenoptik mit Kristallreflektoren. *Naturwissenschaften*, 38 (1951), 250—258.

PART III

- [183] ANDREWS, C. L.: Concave Spherical Crystals of Barium-Copper-Stearate for Use in Long Wave-Length X-Ray Spectrometers. *Rev. Sci. Instr.*, 11 (1940), 111—114.
- [184] BERNSTEIN, S.: Comparison of X-Ray Photographs Taken with X and Y Built-Up Films. *J. Am. Chem. Soc.*, 60 (1938), 1511.
- [185] BIRKS, L. S.: Apparatus for Vacuum X-Ray Fluorescence Analysis of Light Elements. *Rev. Sci. Instr.*, 22 (1951), 891—894.
- [186] — & BROOKS, E. J.: Hafnium-Zirconium and Tantalum-Columbium Systems. Quantitative Analysis by X-Ray Fluorescence. *Anal. Chem.*, 22 (1950), 1017—1020.
- [187] —, — Analysis of Uranium Solutions by X-Ray Fluorescence. *Anal. Chem.*, 23 (1951), 707—709.
- [188] —, —, FRIEDMAN, H., & ROE, R. M.: X-Ray Fluorescence Analysis of Ethyl Fluid in Aviation Gasoline. *Anal. Chem.*, 22 (1950), 1258—1261.

- [189] BLODGETT, K. B.: Films Built by Depositing Successive Monomolecular Layers on a Solid Surface. *J. Am. Chem. Soc.*, 57 (1935), 1007—1022.
- [190] BROGREN, G.: Investigations into X-Ray Absorption Spectra of Rare Gases with Geiger Counters and Bent Crystal. *Nova Acta Regiae Soc., Sci. Upsaliensis, Ser. 4, Vol. 14, No. 4* (1949).
- [191] CASTAING, R., & GUINIER, A.: Point-by-Point Chemical Analysis by X-Ray Spectroscopy. Electronic Microanalyzer. *Anal. Chem.*, 25 (1953), 724—726.
- [192] ENGSTRÖM, A.: Quantitative Cytochemical Determination of Nitrogen by X-Ray Absorption Spectrography. *Biochim. et Biophys. Acta*, 1 (1947), 428—433.
- [193] — Note on the Cytochemical Analysis of Elements by Roentgen Rays. *Acta Radiol.*, 36 (1951), 393—396.
- [194] — & LINDSTRÖM, B.: Histochemical Analysis by X-Rays of Long Wavelengths. *Experientia*, 3 (1947), 191—193.
- [195] — & WEISSBLUTH, M.: Absorption of X-Rays in Inhomogeneous Histo- and Cytological Samples. *Exptl Cell Research*, 2 (1951), 711—714.
- [196] FRIEDMAN, H., & BIRKS, L. S.: A Geiger Counter Spectrometer for X-Ray Fluorescence Analysis. *Rev. Sci. Instr.*, 19 (1948), 323—330.
- [197] GLOCKER, R., & FROHNMAYER, W.: Über die röntgenspektroskopische Bestimmung des Gewichtsanteiles eines Elementes in Gemengen und Verbindungen. *Ann. Physik*, 76 (1925), 369—395.
- [198] HAGLUND, P.: An Experimental Investigation into L-Emission Spectra. *Arkiv Mat. Astron. Fysik*, 28 A, No. 8 (1941).
- [199] v. HEVESY, G., & ALEXANDER, E.: *Praktikum der chemischen Analyse mit Röntgenstrahlen*. Akademische Verlagsgesellschaft m. b. H., Leipzig (1933).
- [200] KOENIG, A. E.: On the Stearates and Palmitates of the Heavy Metals with Remarks Concerning Instantaneous Precipitations in Insulating Solutions. *J. Am. Chem. Soc.*, 36 (1914), 951—961.
- [201] LAURILA, E.: Über die Möglichkeiten der quantitativen Absorptionsanalyse mittels eines Ionisationsspektrometers. *Ann. Acad. Sci. Fennicae, Ser. A, I, No. 6* (1941), 1—14.
- [202] LIEBHAFFSKY, H. A.: X-Ray Absorption. *Anal. Chem.*, 21 (1949), 17—24.
- [203] — X-Ray Absorption. *Anal. Chem.*, 22 (1950), 15—16.
- [204] — X-Ray Absorption in Chemical Analysis and Control. *Ann. N. Y. Acad. Sci.*, 53 (1950—51), 997—1014.
- [205] — X-Ray Absorption. *Anal. Chem.*, 23 (1951), 14—16.
- [206] — X-Ray Absorption. *Anal. Chem.*, 24 (1952), 16—20.
- [207] — Analytical Methods Based upon X-Ray Absorption. *Anal. Chem.*, 25 (1953), 689—692.
- [208] MORTIMORE, D. M., & ROMANS, P. A.: X-Ray Spectroscopy as a Control Method in the Production of Zirconium and Hafnium. *J. Opt. Soc. Amer.*, 42 (1952), 673—677.
- [209] MOXNES, N. H.: Notiz über eine Abänderung des Glockerschen Verfahrens zur quantitativen Analyse mittels Absorption der Röntgenstrahlen. *Z. physik. Chem., A*, 144 (1929), 134—136.
- [210] — Quantitative chemische Analyse mittels der Absorption der Röntgenstrahlen. *Z. physik. Chem., A*, 152 (1930—31), 380—408.
- [211] VOGES, F.: Ein Fortschritt in der quantitativen chemischen Analyse mit Röntgenstrahlen. *Z. Physik*, 80 (1933), 542—556.

Price Sw. Kr. 30:—